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THE ABSORPTION OF SULPHONAMIDES IN THE CHICK AND THE CANARY, AND ITS RELATIONSHIP TO ANTIMALARIAL ACTIVITY

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From the Wellcome Laboratories of Tropical Medicine, London, England

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Investigations on the antimalarial activity of sulphanilamide and its derivatives against *Plasmodium cathemerium* infections in canaries and *P. gallinaceum* in chicks have confirmed previous observations that, while most sulphonamides show a pronounced activity against chick malaria, little or no activity is found against canary infections. It was decided, therefore, to undertake a thorough investigation of the rates of absorption and the blood concentrations attained in chicks and canaries receiving doses of these drugs.

While numerous reports on blood concentrations of sulphonamides in man and other mammals are available, little work has been done on the fate of these drugs in birds. Litchfield (1) reported on the distribution of sulphanilamide in chickens of unspecified age and stated that, when administered orally by stomach tube or in capsules, a large proportion of the dose frequently passed through the gut and was evacuated without being absorbed. Bieter *et al.* (2) give figures for blood concentrations in chickens (8-26 weeks old) for sulphanilamide, sulphapyridine and sulphathiazole. According to this report, the blood concentration of sulphanilamide remains fairly high for over 24 hours, while that of sulphapyridine and of sulphathiazole falls nearly to zero after 12 hours. Blood concentration time curves for sulphonamides were determined in canaries and ducks by E. K. Marshall *et al.* (3), who reported that the drugs disappeared more rapidly from the blood of canaries than of ducks.

It is impossible to collect pure samples of urine from birds. Of the mixed excreta an indeterminate proportion of the recovered sulphonamide would be unabsorbed drug from the gut. No excretion experiments were therefore done on chicks or canaries in the present work. The rate of absorption from the gut was investigated by determining the amount of residual sulphonamide in the guts of birds killed at different times after dosing.

If it is assumed that sulphonamides act directly upon the malaria parasite within the red blood cell, determination of the concentration of drug inside the red cell is important. Red cell concentrations or cell-plasma ratios for sulphonamides have not previously been determined in chicks or canaries. Distribution between whole blood and serum of humans has been determined by Heinemann (4), and red cell concentrations in human blood were investigated by Reinhold *et al.* (5) and by Sise (6). These workers showed that the red cell concentration varied between different derivatives of sulphanilamide and was not necessarily proportional to the distribution of water between plasma and cells, as was at first thought to be the case.

EXPERIMENTAL METHODS. Animals. The chicks used were of a uniform strain (Rhode Island Red crossed with Light Sussex) and were 7-14 days old (50-100 gram body weight). The canaries were of varied strain. Even some birds of a finch variety had to be used, owing to the shortage of canaries. These were not readily infected by *P. cathemerium* and so were used for absorption tests. Canaries surviving antimalarial tests were also used after a few weeks rest. In spite of these difficulties, and also that the scarcity of birds allowed only one to be used for the determination of each point on the curves, no serious individual variation was experienced.

Dosage. A single dose of 250 mgm. of sulphonamide per kgm. body weight was given for all absorption tests. The drugs were finely powdered and suspended in water using a little Compound Powder of Tragacanth to aid dispersion. The suspensions were administered by mouth from a syringe fitted with 8 cm. of thin gutta percha catheter tube (for canaries) or a large bore hypodermic needle with the point cut off (for chicks). In some of the antimalarial tests, drugs were given to canaries incorporated in small pellets each weighing about 10 mgm., a method advocated by Baranger and Thomas (7). The active ingredient was mixed with sufficient powdered glucose to produce the required bulk and massed with a little Tragacanth and Syrup of Liquid Glucose. After dosing, the birds were allowed no food during the period of the test. Those birds which were kept for 16 hours were given drinking water.

The question arose as to the rate of passage of drugs through the gut, and how this was influenced by the method of dosage. By dissection, it was found that the rigid tube method used for chicks deposited the dose in the crop, while the flexible tube used for canaries deposited the dose in the proventriculus. Investigations of the rate of passage of carmine through the gut when administered by the different methods gave the following results:—

Canary 1. One 10 mgm. pellet of carmine. Carmine appeared in the faeces after 1 hour.

Canary 2. 0.5 cc. carmine suspension by flexible tube. Red faeces passed after $\frac{1}{2}$ hour.

Chick 1. Two 10 mgm. pellets of carmine. Carmine appeared in the faeces after $2\frac{1}{2}$ hours.

Chick 2. 1.5 cc. carmine suspension by rigid tube. Red faeces passed after 2 hours.

Chick 3. 1 cc. carmine suspension by soft rubber catheter (into the proventriculus). Red faeces passed after $1\frac{1}{2}$ hours.

These observations show that the rate of passage of carmine through the gut is more rapid in the canary than in the chick. It does not, however, vary greatly with the method of administration, a fact confirmed in antimalarial tests in which both dosage by tube and by pellets has been used.

Determination of sulphonamides in whole blood. The birds were killed in ether vapour, and blood was drawn directly from the exposed heart and run into tubes containing a little potassium oxalate or heparin. The latter was found to be more satisfactory for canary blood, which clots very rapidly. Free and acetylated

sulphonamide was determined by the method of Bratton and Marshall (8) using 0.2 cc. of blood for each determination. Colours were measured in a Hilger Spekker Photoelectric Absorptiometer using a mirror galvanometer. Standard curves were prepared for each sulphonamide derivative by adding known amounts of the drug to blood laked in the same proportion as in the tests.

Determination of sulphonamides in red blood cells. Chicks. The birds were killed in ether vapour, and blood was obtained from the heart and oxalated. The free sulphonamide in 0.2 cc. of whole blood was determined. About 1 cc. of the remaining blood was run into agglutination tubes graduated at 0.1 cc. intervals. The tubes were centrifuged in a Hearson centrifuge at 6000-7000 r.p.m. for 15 minutes (length of angle-arm, 12 cm.). The volume of whole blood and of red cells was noted and the sulphonamide content of the plasma determined, using 0.2 cc. as for whole blood. The sulphonamide content of the red cells was obtained by difference and comparative concentration curves were plotted for whole blood and red cells.

Canaries. Because of the small volume of blood obtainable from these birds, and the readiness with which it clots, the experimental technique had to be modified. Immediately before killing, 0.02 cc. of blood was obtained in a blood pipette after pricking the leg or wing vein. This sample was run directly into the measured volume of water and used to determine whole blood sulphonamide. The canary was then killed. Blood drawn from the heart was heparinized, and about 0.2 cc. run into special micro-centrifuge tubes. These tubes were made by sealing off at one end short lengths of thick-walled manometer tubing (2 mm. bore) and graduating the tubes at intervals of 0.01 cc. Tubes containing blood were centrifuged as above and the sulphonamide content of the plasma determined, using 0.02 cc. When using 0.02 cc. samples, the volumes of water and reagents were reduced accordingly. The volume of final coloured solution was 0.6 cc. and was read in micro-cells of this capacity.

Determination of residual sulphonamide in the gut. The entire gut, including the crop, was removed, ground to a smooth paste with sand, and suspended in 98 per cent alcohol. The suspension was boiled gently on a water-bath for 10 minutes, cooled, made up to known volume with alcohol, and filtered through Whatman No. 1. filter paper. The free and total sulphonamide content of the alcoholic extract was determined as for blood, except that the precipitate formed on adding the trichloroacetic acid, being too fine for removal by centrifuging, was removed by double filtration through Whatman No. 3 (extra thick) paper. Known amounts of sulphonamide added to ground gut were recoverable by this method within an error of ± 6 per cent.

Antimalarial tests. Chicks. Blood from a bird heavily infected with *P. galinaceum* was diluted with normal saline containing 0.1 per cent of "Liquoide" (Roche) to prevent clotting. The dilution was adjusted to contain 100 million parasitized red cells in 0.25 cc. Groups of chicks (7-14 days old) were inoculated intravenously each with 100 million parasitized cells. Dosage was commenced shortly after inoculation, and was continued twice daily for four days. Antimalarial activity was assessed on the fifth day (on which the blood infection of

the control birds reached its peak) by comparison of the percentages of red cells parasitized. The antimalarial tests on chicks were carried out by L. G. Goodwin, to whom the author is indebted for permission to publish the results.

Canaries. Groups of birds were inoculated by injection into the leg vein of diluted canary blood infected with *P. cathemerium*. The technique of intravenous inoculation (instead of the more usual intramuscular route) was in the experimental stages during these tests, and the number of parasitized red cells injected was varied between 1 and 10 million, in order to determine the optimum inoculum. Dosage of the birds, usually by pellets, was commenced shortly after inoculation and continued at twice daily intervals for five days. Daily counts were made of the number of red cells parasitized. Antimalarial activity was assessed by comparing the delay in the appearance of parasites in the blood.

TABLE 1
Antimalarial activity of sulphonamide derivatives

DERIVATIVE	ACTION ON <i>P. GALLINACEUM</i> IN CHICKS (APPROXIMATE SULPHADIAZINE EQUIVALENTS)	ACTION ON <i>P. CATHMERIUM</i> IN CANARIES
Sulphanilamide	0.5	trace
Sulphapyridine	trace	inactive
Sulphadiazine	1.0	inactive
Sulphamezathine	1.0	inactive
Sulphathiazole	0.5	inactive
5-sulphanilamido-quinoline	trace	definite slight activity
8-sulphanilamido-quinoline	trace	inactive
8-sulphanilamido-6-methoxy-quinoline	trace	trace
2-sulphanilamido-benzamide	0.1	inactive
3-sulphanilamido-benzamide	0.2	inactive
4-sulphanilamido-benzamide	0.2	inactive
N'-(6'-quinoxaly)sulphanilamide	0.2	inactive

Nomenclature of drugs tested. The twelve drugs tested were sulphanilamide, sulphapyridine (supplied by Messrs. May & Baker, Ltd.), sulphadiazine, sulphamezathine, sulphathiazole, 5-sulphanilamido-quinoline (Winterbottom (9) and Bobrański (10)), 8-sulphanilamido-quinoline (Winterbottom (9), Bobrański (10) and Choudhury, Das-Gupta and Basu (11)) and 8-sulphanilamido-6-methoxy-quinoline (Choudhury, Das-Gupta and Basu (11)), 2-, 3- and 4-sulphanilamido-benzamides and N'-(6'-quinoxaly)sulphanilamide. The benzamide derivatives were prepared in these laboratories by Dr. J. H. Gorvin (12) and the quinoxaly compound by Dr. B. Platt (unpublished work).

RESULTS. Antimalarial Tests. Table 1 shows the activity of these sulphonamide derivatives against *P. gallinaceum* and *P. cathemerium*. Sulphadiazine and sulphamezathine show considerable activity against chick malaria in doses of 50 mgm. per kgm. body weight, the activity being approximately the same as that of a similar dose of quinine. Sulphanilamide and sulphathiazole show approxi-

mately half the activity of sulphadiazine, and the other sulphonamides show smaller fractions or only a trace.

Against *P. cathemerium* in the canary, only sulphanilamide, 5-sulphanilamido-quinoline and 8-sulphanilamido-6-methoxy-quinoline show a trace of activity in doses of 250–500 mgm. per kgm. body weight. No activity was shown by any of the other derivatives in the dose range given. In two birds which received 40 pellets, each containing 5 mgm. of sulphadiazine, during a period of four days, it was found that the number of parasitized red cells remained at less than 0.5 per cent as long as the dosage was maintained. The infection increased rapidly, however, from the day dosing ceased. The blood concentration of free sulphadiazine in these birds reached peaks of 58 and 107 mgm. per 100 cc. respectively during the test.

Absorption tests. Curves showing the rate of disappearance from the gut, and the blood concentrations attained, after single doses equivalent to 250 mgm. per kgm. body weight, are reproduced in fig. 1 (chicks) and fig. 2 (canaries). Sulphadiazine and sulphamezathine show the highest and most prolonged blood concentration curves, while the sulphanilamido-quinolines appear in the blood in very small concentrations. In both canaries and chicks, sulphanilamide and 5-sulphanilamido-quinoline show the greatest degree of acetylation.

The drugs disappear more rapidly from the gut in canaries than in chicks, probably partly due to the greater rate of passage through the gut in the former, with consequent loss of unabsorbed drug. In canaries, the concentration of drugs inside the red cells is in most cases higher than in the whole blood, particularly in the sulphanilamido-benzamide derivatives. In chicks, the red cell concentrations of sulphadiazine, sulphathiazole, and sulphapyridine are lower than the whole blood concentrations, and, generally speaking, the red cell concentration is lower, compared with the whole blood concentration, than in canaries.

Discussion. It may be assumed that, in order to inhibit the malaria parasite, a drug must come into direct contact with the organism in sufficient concentration for a sufficient length of time. Drugs are administered to the host by mouth and are absorbed from the gut into the blood. Since the schizont resides within the red blood cell, the drug must pass from the plasma into the red cell before coming into direct contact with the parasite. The following factors will therefore influence the final concentration of drug surrounding the parasite; (a) the rate of absorption from the gut into the blood; (b) the rate of excretion of the drug from the blood through the kidneys; (c) the extent to which the drug passes into the red cell from the plasma; (d) the extent to which the drug is converted to inactive derivatives by metabolic processes (e.g. the acetylation of sulphonamides).

In the present investigation, factors (a), (c) and (d) have been studied directly. Information on (b), the rate of excretion, can be deduced from the length of time that the blood concentration is maintained. The question to be answered then, is whether this information can be correlated with the observed activity of these compounds against malaria parasites.

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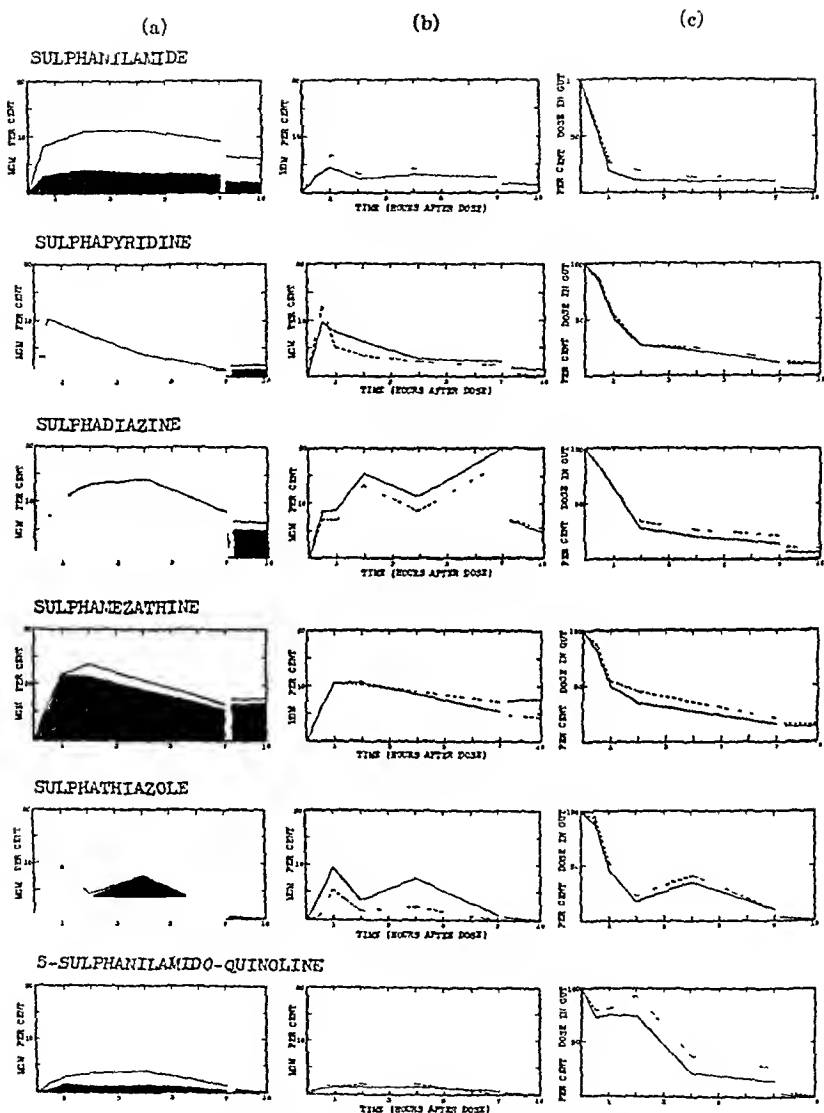


FIG. 1. SULPHONAMIDE DERIVATIVES IN CHICKS

(a) Whole blood concentrations Black areas, free sulphonamide, white areas, acetylated sulphonamide

(b) Comparative concentration curves of free sulphonamide in whole blood (continuous line) and in red blood cells (broken line)

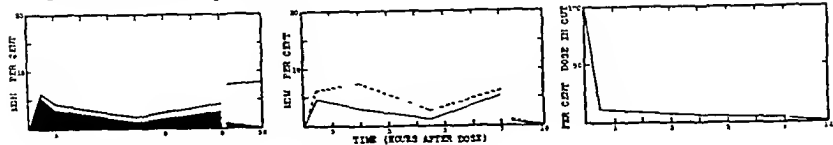
(c) Curves representing percentage of residual sulphonamide in the gut Continuous line, free sulphonamide, broken line, total sulphonamide

(a)

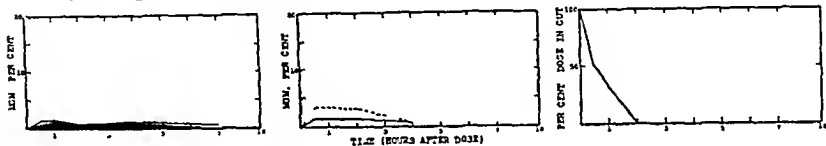
(b)

(c)

8-SULPHANILAMIDO-QUINOLINE



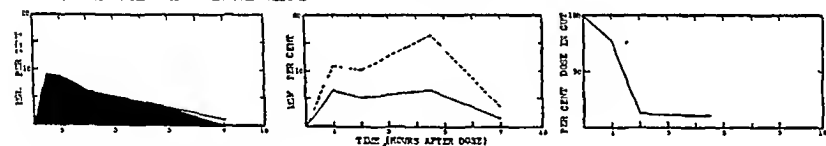
8-SULPHANILAMIDO-6-METHOXY-QUINOLINE



2-SULPHANILAMIDO-BENZAMIDE



3-SULPHANILAMIDO-BENZAMIDE



4-SULPHANILAMIDO-BENZAMIDE



N'-(6'-QUINOXALYL)-SULPHANILAMIDE

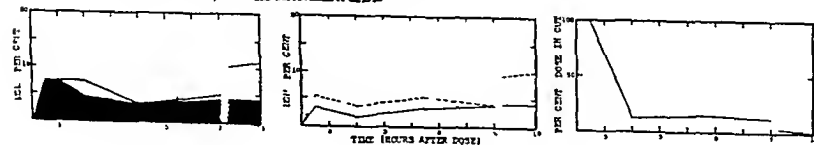


FIG. 1 (continued)

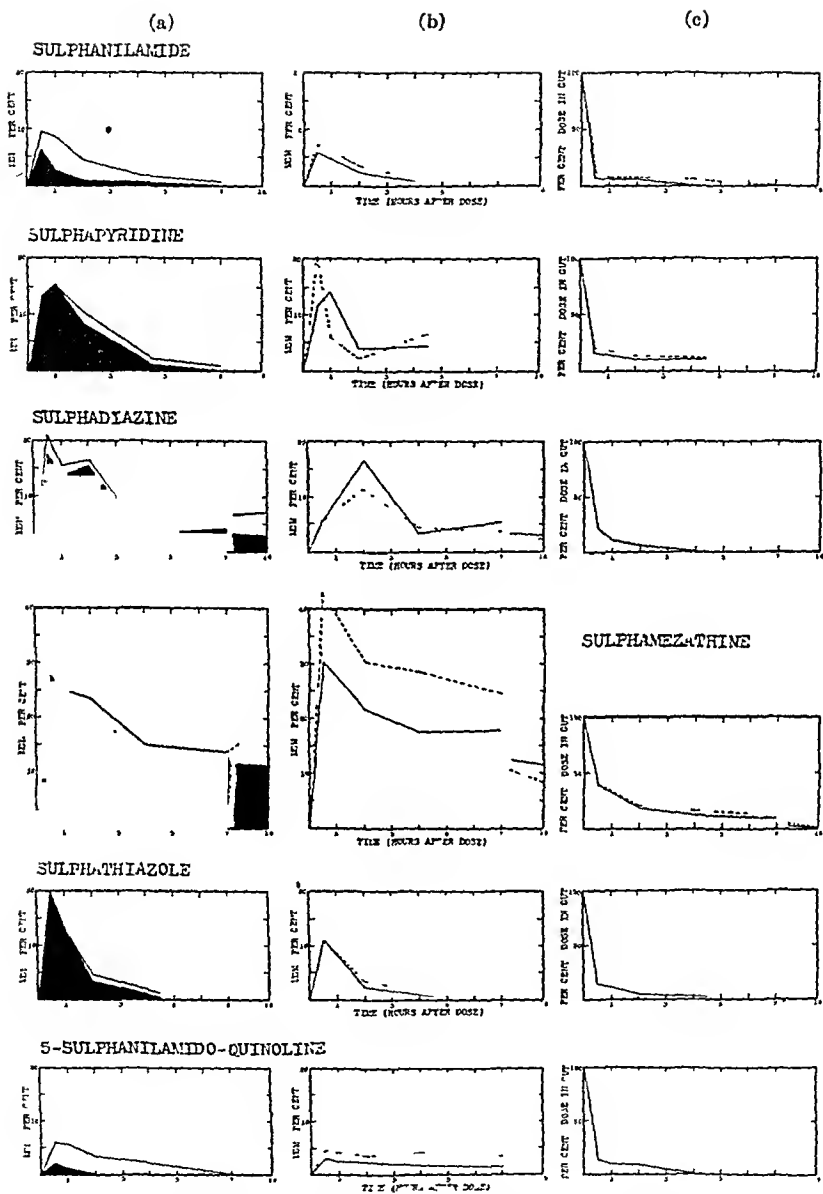


FIG 2 SULPHONAMIDE DERIVATIVES IN CANARIES

(a) Whole blood concentrations Black areas, free sulphonamide, white areas, acetylated sulphonamide

(b) Comparative concentration curves of free sulphonamide in whole blood (continuous line) and in red blood cells (broken line)

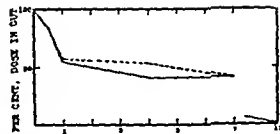
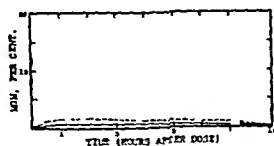
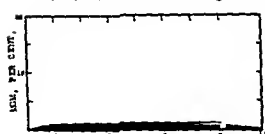
(c) Curves representing percentage of residual sulphonamide in the gut Continuous line, free sulphonamide, broken line, total sulphonamide

(a)

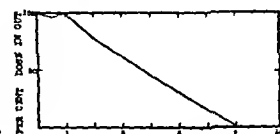
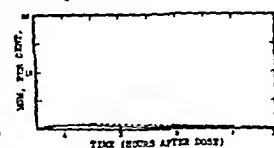
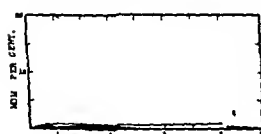
(b)

(c)

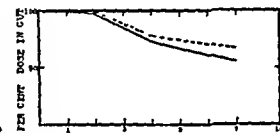
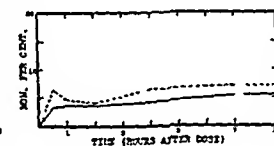
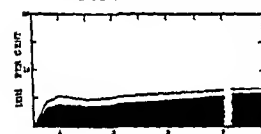
8-SULPHANILAMIDO-QUINOLINE



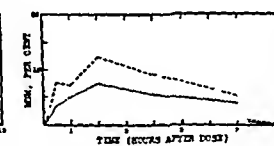
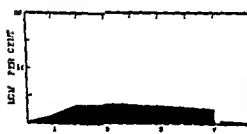
8-SULPHANILAMIDO-6-ETHOXY-QUINOLINE



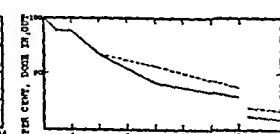
2-SULPHANILAMIDO-BENZAMIDE



3-SULPHANILAMIDO-BENZAMIDE



4-SULPHANILAMIDO-BENZAMIDE



N'-(6'-QUINOXALYL)-SULPHANILAMIDE

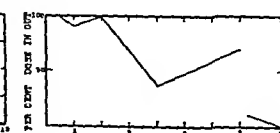
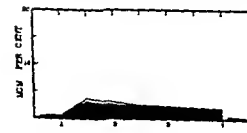


Fig 2 (continued)

trations, which are maintained at a fairly high level for at least 16 hours. In the twice daily dosage regimen, 16 hours is the longest period between any two doses (that is, between the evening and following morning doses). Hence, if a single dose produces an appreciable blood concentration lasting for 16 hours or longer, then, as long as twice daily dosage is maintained, the blood concentration will never fall to zero.

Sulphanilamide and sulphathiazole show about half the antimalarial activity of sulphadiazine, and the blood concentration curves for these compounds are correspondingly lower. The sulphanilamido-benzamides and N'-(6'-quinoxalyl)-sulphanilamide show about one fifth of the activity of sulphadiazine. The blood concentrations are fairly low, and, except in 2-sulphanilamido-benzamide, fall to zero within 16 hours. The three sulphanilamido-quinoline compounds gave very low blood concentration curves, and their antimalarial activity amounted to only a trace. Sulphapyridine shows only slight antimalarial activity, and, though it produces an initial high blood concentration, this falls to a low value within 7 hours,

The red cell concentration in chicks was in most cases the same as, or slightly higher than, the whole blood level. Exceptions were sulphadiazine and sulphathiazole, in which the red cell concentrations were lower; in the case of sulphathiazole, so low as to appear hardly in agreement with the observed antimalarial activity.

On the whole, however, it may be said that the blood concentration curves for these compounds in chicks are in agreement with the order of antimalarial activity.

Turning now to the canary experiments, the red cell concentrations for sulphamezathine and the sulphanilamido-benzamides are much higher than the corresponding chick curves, though the blood concentration curves fall away more rapidly. These drugs are, however, quite inactive against *P. cathemerium* in doses equivalent to the chick doses. It was found, though, that by maintaining canaries on frequently repeated doses of sulphadiazine, so that the whole blood concentration reached peaks of 50 to 100 mgm. per 100 cc., the infection could be suppressed just as long as the dosing was continued. It therefore seems that, in order to inhibit *P. cathemerium* in canaries, very much higher blood concentrations of sulphadiazine must be maintained than are required to inhibit *P. gallinaceum* in chicks. Whether the other sulphonamides would suppress the infection in canaries if given in sufficiently high doses is impossible to determine, since they are all more toxic than sulphadiazine. The trace of activity shown by the sulphanilamido-quinolines might be due to the influence of the quinoline part of the molecule.

In conclusion, therefore, the results obtained from these experiments show that, in chicks, the order of activity against *P. gallinaceum* is on the whole correlated with the order of the blood concentrations attained. In canaries this may or may not be so. Even if it is, the levels at which activity might be obtained are so much higher than the active levels in chicks as to be indeterminable owing to the intervention of toxic action. In comparing the antimalarial effects of sulphona-

mides in chicks and canaries, factors other than those already considered must be involved. It has been shown that the concentration of sulphonamide surrounding the malaria parasite in the canary red cell is, if anything, higher than that surrounding the parasite in the chick red cell. The factors controlling activity or non-activity between chick and canary are therefore probably concerned with the direct action of the drug on the parasite. Whether the variation of effect is dependent upon the different species of host or of parasite can only be determined by further experiment.

SUMMARY

1. Determinations of the absorption from the gut, whole blood concentration curves ("free" and "total") and red cell concentration curves ("free") of sulphanilamide and eleven derivatives in the chick and the canary show that these drugs are absorbed more quickly and are excreted more quickly from the blood in canaries than in chicks. Red cell concentration curves are, in most cases, higher in the canary than in the chick.

2. These drugs show pronounced activity against *Plasmodium gallinaceum* infections in chicks, but are mostly inactive against *P. cathemerium* in canaries.

3. Generally speaking, the degree of antimalarial activity of these drugs in chicks may be correlated with the height of the blood concentration curves. In canaries, similar concentrations are inactive, but there is evidence that *P. cathemerium* would be inhibited if a sufficiently high blood concentration could be maintained.

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A METHOD FOR THE DETERMINATION OF ANALEPTIC ACTIVITY

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The "mouse-awakening test" has been extensively used for the assay of analeptic drugs. The analeptic is injected into the mice either at the same time as an anaesthetic, or after the attainment of a standard depth of anaesthesia. The reduction of duration of the anaesthesia caused by the drug is used as a measure of potency.

We have had occasion to use the test for the assay of stimulant substances (1) and consider that the method we have used for the assessment of analeptic activity may be of interest to others.

EXPERIMENTAL PROCEDURE. The method devised by Chakravarti (2) was chosen as the basis of the assay. Groups of 10 mice were injected intravenously with nembutal (65 mg. per kg.); 0.5 ml. of a solution in saline was given per 20 g. mouse. The time was taken with a stop-watch and recorded. Exactly 20 min. after the dose of nembutal, each mouse was injected subcutaneously with 0.5 ml. of a solution of the drug to be tested, and laid upon its back. The time taken for each animal to turn over into a "sitting" posture was recorded. The time of anaesthesia of a mouse was found to depend upon several external influences: 1, day-to-day variations of susceptibility; 2, temperature; 3, disturbance produced by movements of neighbouring mice; 4, disturbance produced by injection of the analeptic.

To minimise the effects of individual and day-to-day variations, mice of approximately the same weight and of the same strain were used, and were starved over night. As a thermostatically controlled room was not available the anaesthetised mice were placed on a shallow metal tray divided into 40 open compartments, and warmed from below with carbon-filament lamps. The compartments, each measuring 5 x 1.5 inches and 1 inch deep, prevented the mice from being disturbed by movements of their neighbours.

The mechanical stimulation produced by the injection of the dose of analeptic was controlled by the use of a group of mice injected with 0.5 ml. of saline, also given exactly 20 min. after the anaesthetic.

In order to be sure that all conditions were controlled from test to test, one group of mice was always injected with a standard dose of picrotoxin. All doses were given as fractions or multiples of the intravenous L.D.₅₀, determined by Irwin and Cheseman's modification of Kärber's method (3). The intravenous route was used for toxicity tests because this method gives the most precise results. The intravenous toxicity figures are not necessarily proportional to the subcutaneous toxicities, but they furnish a basis upon which the relative analeptic activities of substances may be assessed.

¹ We are indebted to Mr. J. M. Judd and Mr. E. Rogers for valuable technical assistance.

CALCULATION OF RESULTS. The quantal response method used by Chakravarti, in which the percentage of mice awake after a fixed time was plotted against the logarithm of the dose of analeptic administered, does not make full use of the information provided by the test. In a biological assay, the point at which 50% of the experimental animals respond has a lower variance than other points upon the dose-response curve. The data from the analeptic test can be treated to make use of the 50% response by application of the "Time-Mortality Curve" method of Bliss (4). If the probit of the cumulative percentage of mice awake at any time is plotted against the time of anaesthesia, a straight line graph is produced. Fig. 1 shows the results for two groups of 40 mice, one

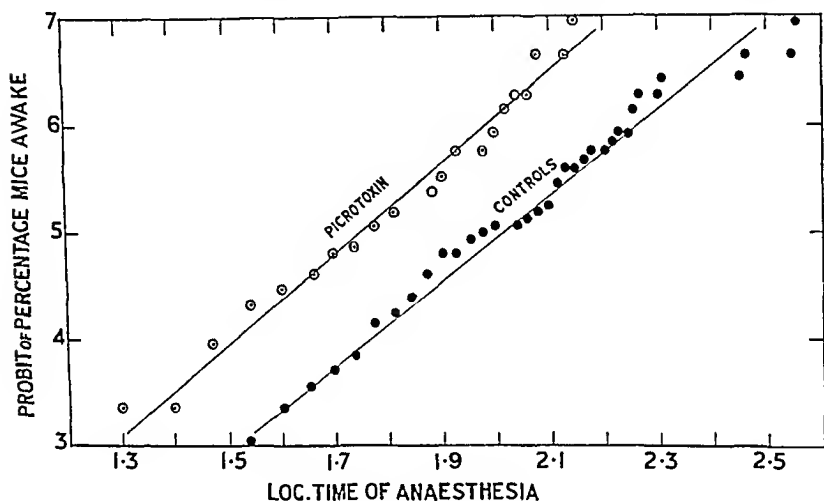


FIG. 1. LOG TIME—PROBIT RESPONSE CURVES FOR THE AWAKENING OF MICE FROM NEMBUTAL ANAESTHESIA

Controls: 40 mice receiving 0.5 ml. per 20 g. 0.85% saline. Picrotoxin: 40 mice receiving 0.045 mg. picrotoxin ($\frac{1}{2}$ intraven. L.D. 50) subcutaneously.

group injected with saline, and the other with picrotoxin. The percentages of mice awake were recorded at 5 minute time intervals. The time taken for 50% of the animals to awaken (the "median anaesthetic time", or "A.T.50") can be determined from the graph.

The A.T.50 for control mice varies from day to day, so that absolute values of the A.T.50 are useless as estimates of the effect of a drug. Accordingly, we have calculated the ratio of the A.T.50 of mice receiving analeptic to the A.T.50 of control mice in each experiment. This "analeptic ratio" when plotted against the logarithm of the dose for a series of experiments yielded the graphs shown in Fig. 2.

RESULTS. The results presented in table 1 show the comparative activities of some well-known stimulant substances against nembutal anaesthesia in mice.

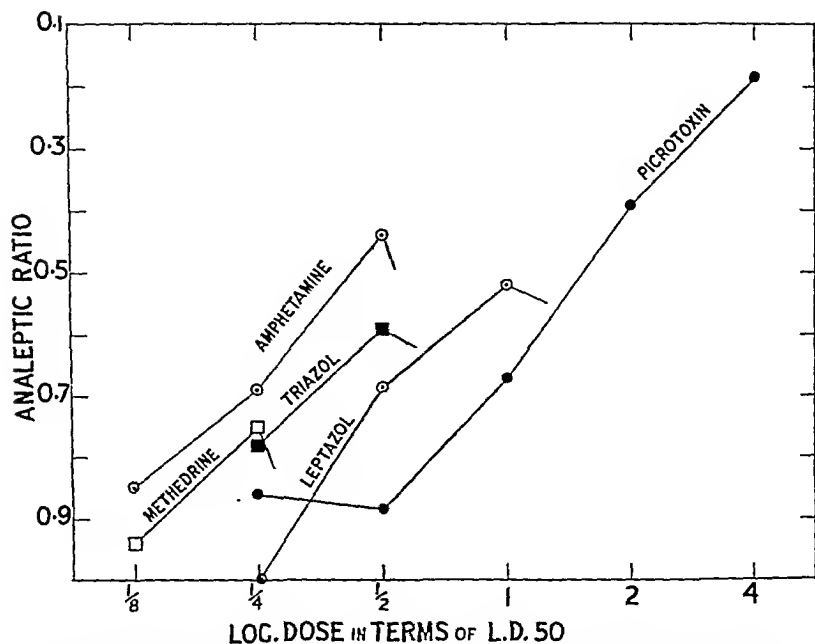


FIG. 2. CURVES SHOWING THE AWAKENING ACTIVITY ON MICE UNDER NEMBUTAL ANAESTHESIA OF DIFFERENT DOSE LEVELS OF FIVE COMMONLY USED STIMULANTS

TABLE 1

The analeptic activity of some familiar stimulants in mice anaesthetised with nembutal

SUBSTANCE	INTRAVE- NOUS L.D. 50	ANALEPTIC RATIO					
		(Dose of compound in terms of L.D.50)					
		1/8	1/4	1/2	1	2	4
Amphetamine sulphate	1.0	0.85 (2)	0.69 (4)	0.44 (2)	0.94 (2)	0.94	—
"Methedrine" hydrochloride	0.50	0.94 (2)	0.75 (2)	P	—	—	—
Leptazol	1.2	—	1.0 (2)	0.69	0.52 (3)	0.61	—
Nikethamide	9.2	—	P	—	P	P	—
Picrotoxin	0.090	—	0.86	0.89	0.68 (21)	0.39 (2)	0.19
Triazol	0.15	—	0.78	0.59	0.78	1.0	—
Strychnine HCl	0.012	—	—	0.79	P	P	—

P = prolongation of anaesthesia. Wherever a figure is the mean of several determinations, the number of experiments is shown in parentheses.

Picrotoxin was by far the most active drug tested, a result which agrees with clinical experience in barbiturate anaesthesia, and confirms the results of other laboratory tests (5, 6, 7).

The other analeptics tested showed toxic effects and prolonged anaesthesia at dose levels of 0.5 to 2 times the intravenous L.D.50, whereas 4 times the L.D.50 of picrotoxin exerted a marked stimulant action. However, doses of this magnitude produced considerable after-effects.

The extent of the day-to-day variation to be expected in the estimate of the analeptic ratio is indicated by the results of 21 determinations for picrotoxin at the L.D.50 dose level, collected over a period of 18 weeks. The mean and standard deviation of these results was 0.68 ± 0.20 .

Nikethamide (coramine) prolonged anaesthesia at all dose levels, although slight respiratory stimulation was occasionally observed.

SUMMARY

1. A modification of Chakravarti's analeptic test is described, in which adequate controls are used in each experiment.

2. The effects of several widely-used stimulants upon mice anaesthetised with nembutal have been compared. The results have been assessed by an application of the "time-response curve" method.

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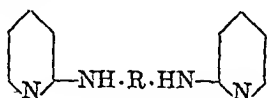
THE PHARMACOLOGICAL PROPERTIES OF SOME MONOAMIDINES

L. G. GOODWIN AND P. B. MARSHALL¹

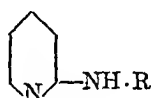
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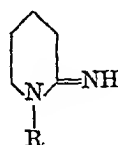
INTRODUCTION. In the search for trypanocidal substances, derivatives of aminopyridine of the types (a), (b) and (c) have been prepared by Sharp (1, 2) and found to be inactive. The group R in (a) represents a chain of from 2 to 10 carbon atoms, and in (b) and (c) an alkyl chain containing from 10 to 14 carbon atoms.



(a)

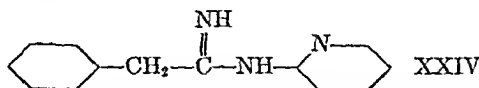


(b)



(c)

Sharp (3) has recently prepared a further series of monoamidine derivatives which we have examined pharmacologically. These substances were designed as possible trypanocides but it was found during toxicity tests in mice that N-2-pyridylphenylacetamidine (XXIV in table 1) in sub-lethal intravenous doses produced an increase in excitability and respiration rate. Large doses caused convulsions. Further tests showed that the convulsions were not associated with hypoglycaemia and that the compound possessed analeptic activity in anaesthetised mice. It was therefore decided to vary the molecule in the hope of producing a useful analeptic.



The length of chain connecting the two rings has been varied, the pyridyl group has been replaced by alkyl groups containing up to 5 carbon atoms and substituents have been introduced into the *meta*- and *para*-positions of the benzene nucleus.

The *m*- and *p*-nitrobenzamidines and aminobenzamidines have previously been prepared by Easson and Pyman (4); the amino compounds were tested as local anaesthetics and found to be inactive. These substances were later tested by Broom (5) for toxicity and for their action upon the blood sugar in the rabbit. The present paper records the results of therapeutic and pharmacological tests made upon the thirty-six amidines listed in table 1. Half of the com-

¹ We wish to thank Mr. T. M. Sharp for the preparation of the compounds, and for his co-operation and advice. We are also indebted to Mr. J. M. Judd and Mr. E. Rogers for valuable technical assistance.

pounds (those containing an N-pyridyl group) were tested quantitatively as analeptics. All have been tested as trypanocides and a selected few have also been tested in canary malaria and hamster leishmaniasis.

METHODS. *Toxicity.* Groups of mice weighing 13–19 g. were injected intravenously with doses of soluble salts of the amidines dissolved in 0.85% saline. A constant volume of 0.5 ml. per 20 g. body-weight was given, and at least 3 groups of 5 or 10 mice were used for each compound. Mortality was observed for 3 days and the L.D.50 and limits of error calculated for each compound by the modification of Kärber's method described by Irwin and Cheeseman (6).

Trypanocidal activity. Mice were infected by intraperitoneal injection of 0.5 ml. of a suspension of *Trypanosoma equiperdum* containing 4,000 organisms per μ l. On the following day, those showing infection in the peripheral blood were injected subcutaneously with large doses of the substance to be tested. The quantity usually given was equivalent to one intravenous L.D.50 which was not lethal when given subcutaneously. In each experiment 5 infected mice were kept as controls and the development of infection in all the mice was followed by daily blood examinations until death.

Other groups of mice were injected intraperitoneally with a suspension of mouse blood containing *Trypanosoma cruzi*. Animals showing parasites in the peripheral blood 10 to 12 days later were grouped in threes and given subcutaneous doses of the compounds at least twice a day, each dose being equivalent to $\frac{1}{2}$ of the L.D.50. Daily blood examinations were made and the course of infection in each mouse compared with that in the untreated controls.

Antimalarial activity. Canaries were inoculated intramuscularly with a suspension of canary blood infected with *Plasmodium cathemerium*. Intramuscular doses of compounds VI and XXXII were given 4 or 5 times a day for six days, the first dose being injected immediately after inoculation. The largest possible doses were given. Blood examinations were made daily and the degree of infection compared with that of control birds.

Leishmanicidal activity. Golden hamsters were inoculated intraperitoneally with a suspension of hamster spleen infected with *Leishmania donovani*. Three months later, the degree of infection of each animal was assessed by the examination of stained smears of biopsied spleen material. Each of the groups of 2 animals were given 5 daily subcutaneous doses of compounds VII, VIII and XVII approaching the toxic level. A second spleen biopsy was made at the end of the treatment and the degree of infection compared with that of the same animal before test (Goodwin (7)).

Action upon smooth muscle. All the compounds were tested upon isolated strips of guinea pig ileum suspended in oxygenated Tyrode solution at 37°C. Compounds XVII, XXVI, and XXXVI were also tested upon ileum contracted by acetylcholine, or with histamine in atropinised Tyrode solution. Their spasmolytic activity was compared quantitatively with that of hexahydrodi-phenylacetyldiethylaminoethanol ester HCl (trasentin-6H).

Action upon the isolated heart. Isolated guinea pig hearts were perfused through the coronary vessels with Ringer-Locke solution at 37°C. Doses of the amidines were added to the perfusion fluid as it entered the aortic cannula.

Action upon blood pressure. The carotid blood pressure of cats or rabbits anaesthetised with nembutal or chloralose was recorded with a mercury manometer and solutions of the amidines injected into the jugular vein. In some experiments the spleen volume was also recorded.

Action upon respiration. The respiration of anaesthetised cats and rabbits was recorded by Gaddum's method (8). Direct action of drugs upon the respiratory centre was dissociated from reflex effects by denervation of the carotid sinuses and section of the vagi.

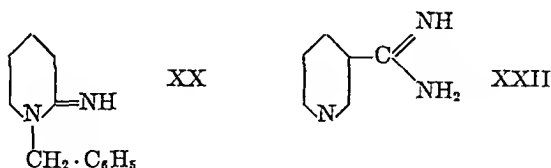
Action upon the knee-jerk reflex. In cats under light nembutal or chloralose anaesthesia, the knee-jerk was produced and recorded by the method of Schweitzer and Wright (9). The effects of intravenous injections of the amidines were compared with the effects of common analeptics.

Awakening action upon anaesthetised mice. The action of the compounds upon mice anaesthetised with nembutal was tested by the method described in an earlier communication, Goodwin and Marshall (10)).

RESULTS. The results of the toxicity tests are shown in table 1.

None of the compounds tested showed trypanocidal, antimalarial or leishmanicidal activity.

Relaxed guinea pig ileum was contracted by concentrations of about 3×10^{-5} of N-methylphenylacetamidine (X), *p*-nitrophenylacetamidine (XV), N-2-pyridyl- β -phenylpropionamidine (XXV) and N-2-pyridyl- γ -phenylbutyramidine (XXVI). The other compounds did not cause contraction in concentrations of less than 10^{-2} . All the compounds except 4-*p*-nitrophenoxy-N-2-pyridylbenzamidine (XXXIV) pyridine-3-carbonamidine (XXII) and N-benzyl-2-pyridoneimine (XX) caused relaxation of the histamine-contracted ileum at concentrations of 3×10^{-4} .



4-*p*-nitrophenoxybenzamidine (XVII) was the most active spasmolytic amidine, but it had only $\frac{1}{10}$ of the activity of trasentin-6H against histamine spasms of the ileum in atropinised Tyrode solution, and $\frac{1}{100}$ of the activity of trasentin-6H against spasms produced by acetylcholine. Most of the compounds reduced the amplitude of the beat of the isolated heart (fig. 1 (A), (B), (C), (D)) but stimulation occurred with phenylacetamidine (IX) (Fig. 1 (E)), *p*-nitrophenylacetamidine (XV), N-methylphenylacetamidine (X), and α -phenylbutyramidine (XVIII). The depressant action of the series XXIII to XXVI increased with increasing molecular weight. The effects of XVIII (stimulant) and XXV (depressant) were not abolished by atropine.

The two pyridoneimines (XX) and (XXI) and pyridine-3-carbonamidine

(XXII) always caused a rise of blood pressure in doses of 2 to 5 mg. per kg., and N-2-pyridyl-*m*-nitrobenzamidine (XXXII) usually did so. The rest of

TABLE 1

The toxicity of a series of monoamidines (intravenous injection into mice)

COMPOUND	REF. NO.	L.D. 50 (MG. BASE PER 20 G. BODY-WEIGHT)	% LIMITS OF ERROR (P = 0.95)
<i>m</i> -chlorobenzamidine HNO ₂ ..	I	2.2	84-119
<i>p</i> -chlorobenzamidine HCl	II	1.8	86-116
<i>m</i> -aminobenzamidine HCl ..	III	6.3	85-118
<i>p</i> -aminobenzamidine HCl	IV	4.5	86-116
<i>p</i> -hydroxybenzamidine HCl	V	5.4	86-116
<i>m</i> -nitrobenzamidine HCl	VI	0.93	86-116
<i>p</i> -nitrobenzamidine HCl	VII	2.1	80-126
3:4-dihydroxybenzamidine HCl	VIII	4.4	92-110
phenylacetamidine HNO ₂	IX	0.93	87-115
N-methylphenylacetamidine HCl	X	0.83	87-115
N-propylphenylacetamidine HCl	XI	0.28	78-128
N-butylphenylacetamidine HCl	XII	0.26	79-127
N-amyphenylacetamidine HCl	XIII	0.32	80-125
N-N'-dimethylphenylacetamidine HCl	XIV	0.39	85-117
<i>p</i> -nitrophenylacetamidine HCl	XV	2.9	92-109
<i>p</i> -aminophenylacetamidine HCl	XVI	3.2	84-119
4- <i>p</i> -nitrophenoxybenzamidine HCl	XVII	1.9	83-120
α -phenylbutyramidine HCl	XVIII	0.20	88-114
2-benzylaminopyridine HCl	XIX	1.8	85-118
N-benzyl-2-pyridoneimine $\frac{1}{2}$ H ₂ SO ₄	XX	0.32	85-118
N- β -phenylethyl-2-pyridoneimine $\frac{1}{2}$ H ₂ SO ₄	XXI	0.17	92-109
pyridine-3-carbonamidine HCl	XXII	3.8	88-114
N-2-pyridylbenzamidine HCl	XXIII	0.61	79-127
N-2-pyridylphenylacetamidine HCl	XXIV	0.60	89-113
N-2-pyridyl- β -phenylpropionamidine HCl	XXV	0.59	87-115
N-2-pyridyl- γ -phenylbutyramidine HCl	XXVI	0.40	84-119
N-2-pyridyl- <i>m</i> -chlorobenzamidine HCl	XXVII	1.3	85-117
N-2-pyridyl- <i>p</i> -chlorobenzamidine HCl	XXVIII	0.98	85-117
N-2-pyridyl- <i>m</i> -aminobenzamidine HCl	XXIX	1.5	86-117
N-2-pyridyl <i>p</i> -aminobenzamidine HCl	XXX	1.6	85-118
N-2-pyridyl- <i>p</i> -hydroxybenzamidine HCl	XXXI	1.9	92-109
N-2-pyridyl- <i>m</i> -nitrobenzamidine HCl	XXXII	1.4	86-116
N-2-pyridyl <i>p</i> nitrobenzamidine HCl	XXXIII	3.6	90-111
4- <i>p</i> -nitrophenoxy-N-2-pyridylbenzamidine HCl	XXXIV	0.65	86-116
N-2-pyridyl- <i>p</i> -nitrophenylacetamidine HCl	XXXV	0.85	85-117
N-2-pyridyl β - <i>p</i> -nitrophenylpropionamidine HCl	XXXVI	0.56	86-117

the compounds produced a profound fall. No significant effect was observed upon the spleen volume.

Most of the pyridyl derivatives stimulated the respiration. Stimulation was caused partly by the reflex effects of the fall of blood pressure, but considerable stimulation occurred in animals with denervated carotid sinuses and

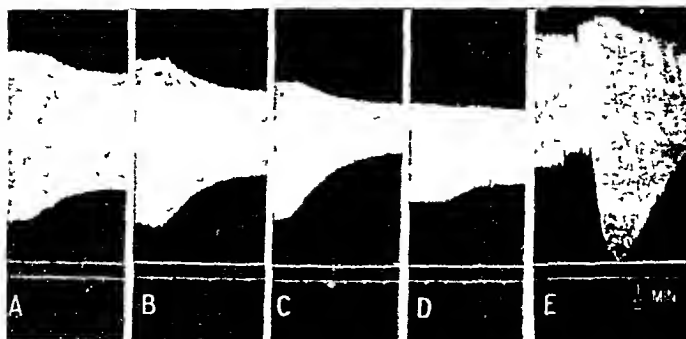


FIG 1 ACTION OF MONOAMIDINES ON THE ISOLATED PERFUSED GUINEA PIG HEART
Downstrokes = systolic At (A) and (D), 0.05 mg N 2 pyridylphenyl acetamide [XXIV] At (B) and (C) 0.05 mg N 2 pyridylphenyl propionamide [XXV] At (E), 0.5 mg phenylacetamide [X]

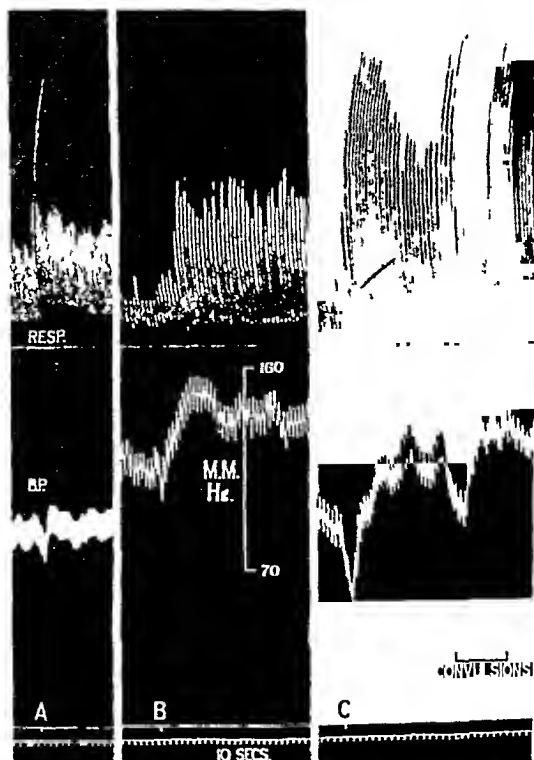


FIG 2 THE EFFECT OF N 2 PYRIDYL *m* NITROBENZAMIDE [XXXII] UPON THE BLOOD PRESSURE AND RESPIRATION OF A NEMBUTALISED CAT (2.5 Kg)

At (A) and (B) 10 mg of N 2 pyridyl *m* nitrobenzamide intravenously At (C) 50 mg N 2 pyridyl *m* nitrobenzamide intravenously Between (A) and (B), the carotid sinuses were denervated and the vagi cut

cut vagi, showing that central activity was also present. Fig. 2 shows the effect of compound XXXII which caused respiratory stimulation and a rise of blood pressure. Pyridine-3-carbonamidine (XXII) caused no stimulation in animals anaesthetised with nembutal or chloralose, and N-2-pyridylbenzamidine (XXIII) was slightly depressant, but the N-2-pyridylphenylacet-, propion-, and butyramidines (XXIV, XXV and XXVI) were all strongly stimulant. Fig. 3 shows the effect of compounds XXIV and XXV upon the respiration and blood pressure of a decerebrate cat; the respiratory effect of XXV is about

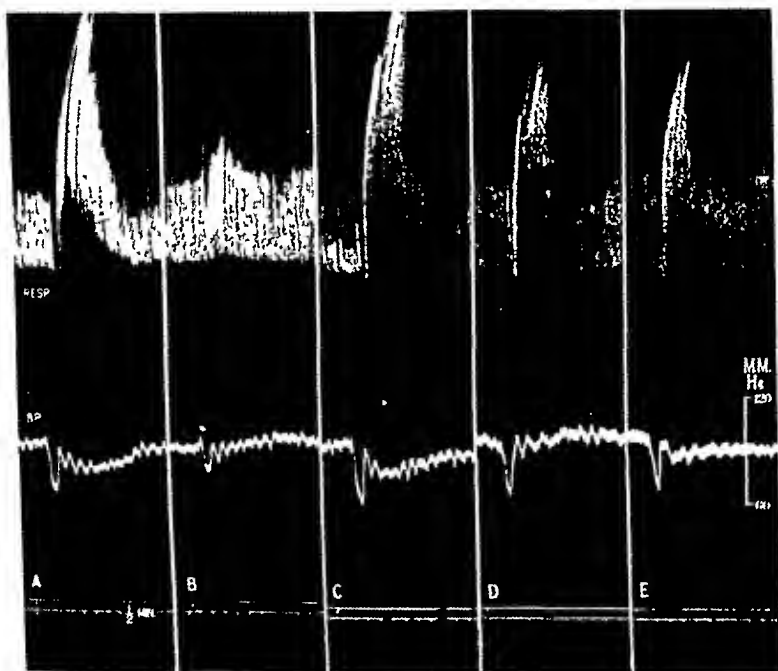


FIG. 3 ACTION OF PYRIDYL MONOAMIDINES ON RESPIRATION AND BLOOD PRESSURE

Decerebrate cat, 2.5 kg. A. [XXV] intravenously. B. [XXIV] intravenously. C. [XXV] intravenously. D. [XXV] intravenously. E. [XXV] intravenously. 3.25 mg N-2-pyridyl- β -phenylpropionamidine [XXV] intravenously.

twice that of XXIV. Compound XXVI was less active than XXV. Compound XXIV produced marked respiratory stimulation in a rabbit deeply depressed with morphine. Depression of the knee-jerk reflex always occurred upon injection of the monoamidines (fig. 4). Analeptics such as leptazol, triazol, picrotoxin and amphetamine always caused enhancement. Nikethamide had no effect.

The analeptic action of the pyridyl compounds in mice anaesthetised with nembutal is shown in table 2 and fig. 5. All the active compounds tested showed

low activity at low dosage, an intermediate effective range and a toxic level at which anaesthesia was prolonged. The four pyridylamidines shown in fig. 5 were the most active, but they were effective over a much narrower range of

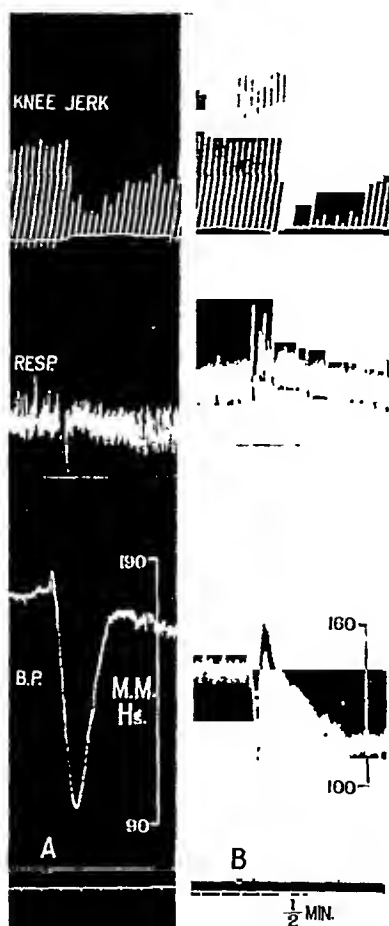


FIG. 4. TYPICAL ACTION OF MONOAMIDINES ON THE KNEE-JERK, RESPIRATION AND BLOOD PRESSURE OF NEMBUTALISED CATS.

At (A), 5.0 mg. phenylacetamidine [IX]. At (B), 10.0 mg. N-2-pyridyl *p*-nitrophenyl-acetamidine [XXXV].

dose than the analeptics reported in our previous paper (10). N-benzyl-2-pyridoneimine (XX) was the most active substance at low dose levels but its action was erratic.

COMMENTARY. These results do not suggest any therapeutic use for the monoamidines, but the compounds show several interesting features. The results of

the therapeutic tests indicate that a single amidine grouping is insufficient to confer trypanocidal or leishmanicidal activity. The results of the recent extensive work on the diamidines show that substances containing two amidine groups are highly active.

The toxicity in short homologous series of monoamidines (IX to XIII, and XXIII to XXVI) increased with increasing molecular weight, and the addition of the pyridyl group always increased the toxicity. The results for the *meta* and *para*-benzamidine derivatives were in general agreement with the findings

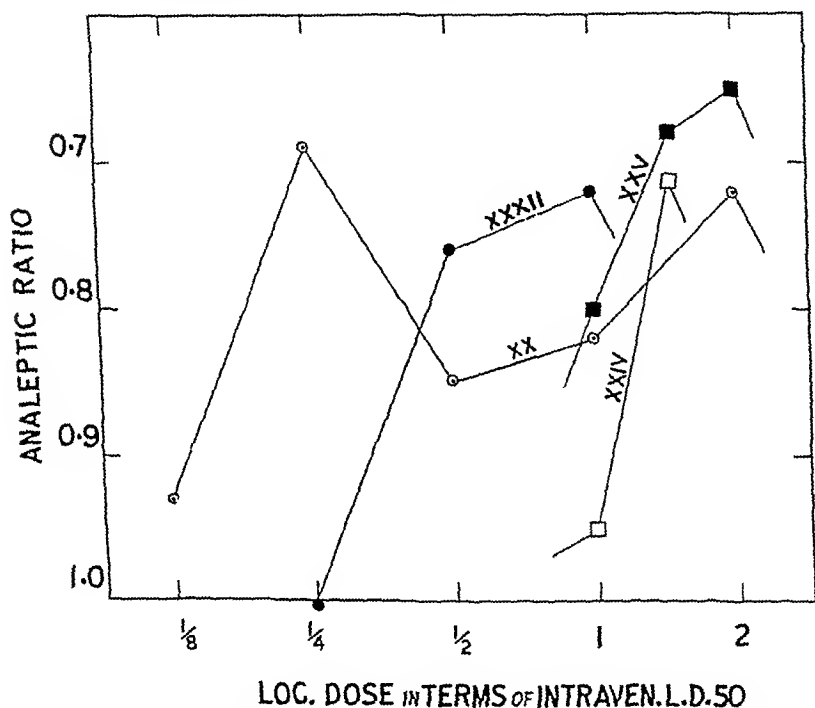


FIG. 5. CURVES SHOWING THE AWAKENING ACTIVITY ON NEMBUTALISED MICE OF FIVE PYRIDYL MONOAMIDINES

of Broom (5), who states that *meta* derivatives are less toxic than *para* derivatives. A notable exception was the case of the N-2-pyridylnitrobenzamidines (XXXII and XXXIII), where the *meta* compound was the more toxic.

The fall in blood pressure produced by most of the compounds was caused partly by depression of the heart muscle. In this, the monoamidines differ from the diamidines, which according to Wien (11) have but a transient and variable action upon the heart.

Nembutal was chosen as the standard anaesthetic for the experiments upon respiratory stimulation and anaesthetic activity. A few experiments were made upon decerebrate cats in the examination of compounds XXII to XXVI, and

TABLE 2

The anaesthetic activity of the pyridyl monoamidines in mice under nembutal anaesthesia

COMPOUND	REF. NO	ANAESTHETIC RATIO						
		Dose of compound in terms of intraven. L.D. ₅₀						
		0.125	0.25	0.50	1.0	1.5	2.0	4.0
2-benzylaminopyridine HCl.	XIX	—	—	P	P	—	P	—
N-benzyl-2-pyridoneimine ½ H ₂ SO ₄	XX	0.93 (4)	0.69 (3)	0.85 (3)	0.82 (2)	—	0.72	Toxic
N-β-phenylethyl-2-pyridone- imine ½ H ₂ SO ₄	XXI	0.83 (3)	P	P	0.63 (2)	—	P	Toxic
Pyridine-3-carbonamidine HCl	XXII	—	—	P	0.96	—	P	—
N-2-pyridylbenzamidine HCl	XXIII	—	—	P	0.80 (2)	—	P	—
N-2-pyridylphenylacet- amidine HCl	XXIV	—	—	P	0.95 (2)	0.71 (2)	P	—
N-2-pyridyl-β-phenyl- propionamidine HCl	XXV	—	—	P	0.80 (5)	0.68 (4)	0.65	Toxic
N-2-pyridyl-γ-phenylbutyr- amidine HCl	XXVI	—	P	0.83	P	—	P	P
N-2-pyridyl- <i>m</i> -chlorobenz- amidine HCl	XXVII	—	—	P	1.0	—	P	—
N-2-pyridyl- <i>p</i> -chlorobenz- amidine HCl	XXVIII	—	—	P	0.72	—	P	—
N-2-pyridyl- <i>m</i> -aminobenz- amidine HCl	XXIX	—	—	P	0.71	—	P	—
N-2-pyridyl- <i>p</i> -aminobenz- amidine HCl	XXX	—	—	P	P	—	P	—
N-2-pyridyl- <i>p</i> -hydroxybenz- amidine HCl	XXXI	—	—	P	P	—	0.79	—
N-2-pyridyl- <i>m</i> -nitrobenz- amidine HCl	XXXII	—	1.0	0.76 (2)	0.72 (5)	—	P	—
N-2-pyridyl- <i>p</i> -nitrobenz- amidine HCl	XXXIII	—	—	1.0	P	—	—	—
4- <i>p</i> -nitrophenoxy-N-2- pyridylbenzamidine HCl	XXXIV	—	—	P	P	—	P	—
N-2-pyridyl- <i>p</i> -nitrophenyl- acetamidine HCl	XXXV	P	0.89	0.91 (2)	P	—	—	—
N-2-pyridyl-β- <i>p</i> -nitrophenyl propionamidine HCl	XXXVI	—	—	1.0	P	—	0.86 (2)	Toxic

P = prolongation of anaesthesia. Wherever a figure is the mean of several determinations, the number of experiments is shown in parentheses.

showed essentially the same type of result as anaesthetised animals. Pyridine-3-carbonamidine (XXII) was disappointing, for it is not unlike nikethamide in configuration. No stimulant action was shown even in chloralose anaesthesia where the respiratory effects of nikethamide were considerable.

In the homologous series XXIII to XXVI, stimulant activity increased with molecular weight as far as the propionamidine (XXV). A further increase in the length of the chain to the butyramidine (XXVI) decreased the activity. Introduction of nitro groups did not appreciably affect the acute toxicity and generally enhanced the analeptic activity of the pyridyl derivatives. Introduction of amino, chloro and hydroxy groups did not increase activity.

The three compounds XIX, XX and XXI are interesting in that they are derivatives of aminopyridine (Sharp (2)) and therefore may be regarded as cyclic amidines. N-benzyl-2-pyridoneimine (XX) was the only one of these to show appreciable, though erratic, activity in anaesthetised mice.

N-2-pyridyl-*m*-nitrobenzamidine (XXXII) was perhaps the most effective pyridyl derivative tested, because it produced a rise of blood pressure as well as respiratory stimulation, and possessed considerable activity in anaesthetised mice. Reduction of the nitro group to the amine (XXIX) rendered the substance less active.

The depression of the knee-jerk which was produced by all the pyridyl amidines shows that their central action differed from that of the well-known analeptics. All of the latter except nikethamide caused stimulation of the reflex.

SUMMARY

1. Some pharmacological properties of a series of 36 monoamidines have been studied.
2. None of these compounds had any action upon infections of *Trypanosoma equiperdum* or *Trypanosoma cruzi* in mice, and a few selected compounds were inactive against hamster leishmaniasis and canary malaria.
3. Pyridyl monoamidines in general showed respiratory stimulation and analeptic activity in anaesthetised animals.
4. Most of the monoamidines caused a fall of blood pressure upon intravenous injection, had spasmolytic activity, and also produced a depression of the knee-jerk reflex.
5. It is unlikely that any of the compounds would be of value in therapeutics.

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STIMULATION AND DEPRESSION OF THE CENTRAL NERVOUS SYSTEM BY DERIVATIVES OF BARBITURIC AND THIOBARBITURIC ACIDS¹

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One of the oldest and most controversial problems of pharmacology is the nature of the "stimulation" appearing in the course of action of central nervous system depressants. Whether the phenomenon is one of direct excitation, or release from inhibition, has been discussed in the case of alcohol, and the inhalation anesthetics, and recently the barbituric acid derivatives. One of these, 1-methylpropyl, 2-bromoallyl barbituric acid, "Pernocton," seemed particularly effective in producing a state of mental and bodily hyperactivity (1), although it became evident that other barbiturates acted similarly (2).

Many barbiturates have been observed in animals to produce such effects as preanesthetic excitement, convulsions during anesthesia, or increased reaction to painful stimuli when given in subanesthetic doses (3-5, 8-11, 13-17, 29, 30). The description by Swanson (5) of the powerful convulsant action of 5,5 (1,3-dimethylbutyl) ethyl barbituric acid suggested further inquiry into the nature of this action, and the chemical structure with which it is associated. To afford information about the simpler central nervous mechanisms, quantitative recording of spinal reflexes was used.

EXPERIMENTS. Fifteen "spinal" dogs were prepared by transection of the spinal cord at the atlanto-occipital space during ether anesthesia. In some cases, the cord was also cut at the tenth thoracic segment. Artificial respiration was supplied. Two dogs were decerebrated with a knife, two cats were decerebrated with a guillotine. Four dogs under anesthesia with a barbiturate, one with chloralose, were used. One hind leg was immobilized with drills through bones, and leg muscles not used were denervated or cut. *M. tibialis anticus* and *m. gastrocnemius* were connected with isometric levers of the bell-crank or torsion-wire type, which wrote on a smoked drum. Nerves for stimulation (femoral, popliteal, tibial) were placed in shielded tubular electrodes and received stimuli from electron-tube devices of the relaxation-oscillator type. In recording the knee-jerk, the foot was connected with an isotonic lever, and the tendon tapped with a solenoid hammer. Pressure in the carotid artery was recorded by a Hg manometer. The drugs were injected into a femoral or jugular vein. Those not obtained as sodium salts were dissolved in a minimal amount of NaOH solution.

RESULTS. The flexion reflex was elicited by applying single maximal or sub-maximal shocks to a sensory nerve at a rate of about thirty per minute. It was recorded in *m. tibialis anticus*. It was augmented, never depressed, in spinal, decerebrate, or barbitalized animals by doses of 1 to 5 mgm. per kgm. of 5,5

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(1,3-dimethylbutyl) ethyl B.A. Larger doses caused convulsions, during which it was not possible to observe the reflex. This compound is more active than caffeine, about one-fourth as active as picrotoxin. It is 250 times weaker than strychnine. It has no influence on the contraction of skeletal muscle elicited by stimulation of its motor nerve, when given in doses which augment the flexion reflex.

The crossed-extension reflex was recorded in *m. gastrocnemius*. The injection of this barbituric acid augmented the rate at which tension was increased, and the height to which it rose.

The knee-jerk, in anesthetized dogs, was decreased following 2 mgm. per kgm., and was increased after 5 mgm. per kgm. of this barbituric acid. The same phenomenon, of reversal of effect with dose, was seen with strychnine.

TABLE 1

1,3-DIMETHYLBUTYL ETHYL BARBITURIC ACID	MORTALITY RATIOS			
	Rats		Rabbits	
	Intraperitoneal injection	Intravenous injection	Intraperitoneal injection	Intraperitoneal injection*
mgm per kgm				
5		0/5		
10		2/6		
20	1/5	3/5	0/5	
25			3/10	
30	3/10	3/3	7/11	0/3
40	4/6			
50				1/3
75				5/10
100				6/8

$$\text{Mortality ratio} = \frac{\text{Number of animals dying}}{\text{Number of animals used}}$$

* Immediately following the intravenous injection of Amytal, 40 mgm per kgm

ANTAGONISMS. In the intact animal, 1,3-dimethylbutyl ethyl B.A. produces increased alertness, running about, and convulsions, as the dose is increased. Death follows violent convulsions, with no stage that might be called depression appearing. Table 1 shows the lethal doses for rats and rabbits, in fair agreement with Swanson and Chen (6).

Previous administration of a depressant barbiturate, Amytal, antagonizes the convulsant action of 1,3-dimethylbutyl ethyl B.A., and raises the fatal dose about three times, when the latter is injected intraperitoneally (table 1). When this stimulant B.A. was injected intravenously in a dose of 30 mgm. per kgm., in two rabbits that had received pentobarbital 25 mgm. per kgm., both died, confirming Swanson and Chen (6).

The reverse antagonism is not marked. An intraperitoneal injection of 100 mgm per kgm. of pentobarbital is uniformly fatal in rabbits (confirming (7)). Although brief lightening of narcosis was seen, a fatal outcome could not be

prevented, in any of seven rabbits receiving the above dose, with intravenous injections of 1,3-dimethylbutyl ethyl B.A. in doses of 4 to 15 mgm. per kgm., in various divisions. Pierotoxin was found (7) to be an effective analeptic in the same circumstance.

RESPIRATORY ACTIVITY. In a barbitalized dog, respiration being recorded with a pneumograph or spirometer, a dose of 1,3-dimethylbutyl ethyl B.A. which augments reflex activity causes a brief but intense hyperpnea, followed by apnea, then by a period of irregular but augmented respiration. It is less effective than picrotoxin as a respiratory stimulant.

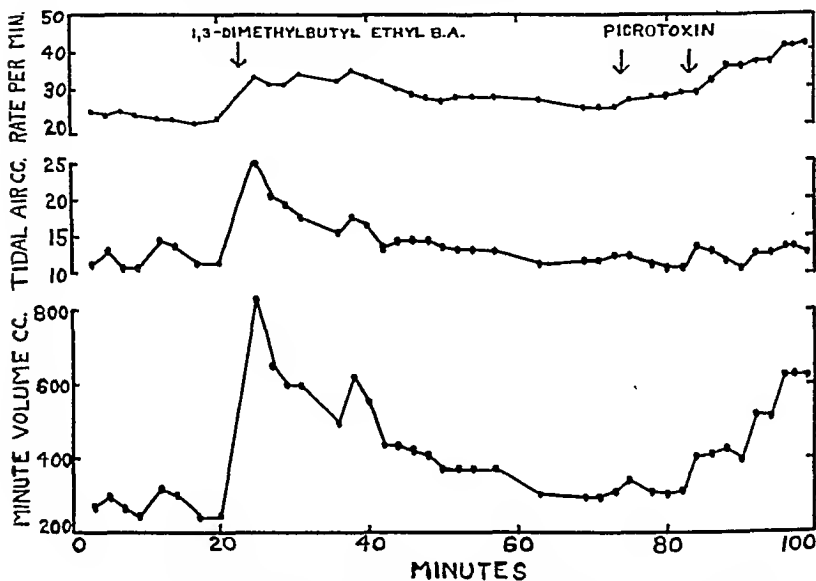


FIG. 1. RABBIT, MORPHINE SULFATE, 5 MGm. PER KGm. SUBCUTANEOUSLY 90 MINUTES BEFORE THE BEGINNING OF THE RECORD

From the top down: rate of respirations per minute; tidal air in cc.; minute volume in cc. Intravenous injections. At the first arrow, 1,3-dimethylbutyl ethyl B.A., 1 mgm. per kgm. At the second arrow, 0.2 mgm. per kgm., at the third arrow, 0.4 mgm. per kgm. of picrotoxin.

The stimulant action of this compound on respiration depressed by the administration of 5-10 mgm. per kgm. of morphine sulfate was studied with the Dreser method in four rabbits. The effect was about equal to that of picrotoxin (fig. 1).

THE ACTION OF RELATED COMPOUNDS. A purely depressant action on the flexion reflex was seen with the closely related compounds, 1-methylbutyl ethyl B.A. (pentobarbital), 3-methylbutyl ethyl B.A. (Amytal), 1-methylpentyl ethyl B.A., 1,3-dimethylpentyl ethyl B.A., and 1,4-dimethylpentyl ethyl B.A.

1,3-dimethylbutyl ethyl thiobarbituric acid, intravenously in rabbits, produces narcosis, but preceding and following the narcosis, there is a stage during which muscular hyperactivity is present. With minimal effective doses, this

may be simply twitching; if the dose is larger, running movements and twitching are seen, followed by narcosis, and death occurs after deep narcosis as seen with purely depressant barbiturates. The flexion reflex in the spinal and decerebrate animal is only depressed by this compound.

COMPOUNDS WITH A DOUBLE BOND. 1-ethylbutenyl-2 ethyl B.A. has been reported to produce anesthesia with convulsions in rats (8). Injected intravenously in rabbits, this compound produces a violent convulsive attack, which is followed by a state resembling the narcosis from a depressant barbiturate, but twitching of the limb muscles and twisting and turning of the head are frequent. Doses up to 20 mgm. per kgm. in spinal and decerebrate dogs had either no effect on the flexion reflex, or depressed it. The same results were seen with the corresponding saturated compound, 1-ethylbutyl ethyl B.A.

1,3-dimethyl-1-butenyl methyl and 1,3-dimethyl-1-butenyl ethyl B.A. have been described as causing convulsions (9). These two substances have been shown to augment the flexion reflex. The methyl compound is much weaker than the ethyl compound, as is found with purely depressant barbiturates. The 1,3-dimethyl-1-butenyl B.A. is less active than the corresponding butyl compound, and the same comparison holds for the 1-methylbutyl and 1-methylbutenyl compounds which are purely depressant. On intravenous injection in rabbits, 1,3-dimethyl-1-butenyl ethyl B.A. has a stimulant, convulsive action resembling that of the butyl compound.

Several barbiturates and thiobarbiturates containing the 2-methylallyl group have been described as convulsant (10), when injected in rats. The following phenomena are seen with the flexion reflex. n-propyl methallyl and allyl methallyl B.A. are purely depressant, the former being the more potent. Di-methallyl B.A. is stimulant in doses of 25 mgm. per kgm., but depressant if more is given. n-propyl methallyl and n-butyl methallyl thiobarbituric acids are about equally stimulant. 1-methylpropyl methallyl and 1-methylbutyl methallyl thiobarbituric acids are about equally stimulant, but considerably more active than the propyl and butyl compounds.

1-methylbutyl ethyl thio B.A. (Pentothal), isopropyl bromallyl B.A. (Noctal) and 1-methylpropyl bromallyl B.A. (Pernocton) caused only depression of the flexion reflex.

COMPOUNDS WITH PHENYL GROUPS. 5-benzyl B.A. has been reported to be inactive (11). The corresponding thio B.A. is not highly active; 100 mgm. per kgm. gave slight augmentation of the flexion reflex, 270 mgm. per kgm. depressed it. Benzyl ethyl B.A. has been described as convulsive in rats, convulsive during anesthesia in cats (11). The spinal flexion reflex was slightly augmented by doses of 50-75 mgm. per kgm. Phenyl ethyl B.A. (Phenobarbital) and phenethyl ethyl B.A. (12) were purely depressant to the flexion reflex, but phenethyl ethyl thio B.A. was a powerful stimulant. It has been described as producing convulsions and no anesthesia in rats (13). Diphenyl barbituric acid had no action on spinal reflexes, in a dose of 25 mgm. per kgm., which is about the maximum tolerated dose for single intravenous injection. Dibenzyl B.A. has been reported to give convulsions during anesthesia in mice (14).

NITROGEN-SUBSTITUTED COMPOUNDS. Many of these have been described

as having stimulant properties (12, 14-17). Only two have been included in this study, p-chlorophenyl and p-bromophenyl barbital. They were not highly active on the flexion reflex, but showed at times slight augmentation at doses around 50 mgm. per kgm.

DISCUSSION. It is apparent that the spinal flexion reflex may be augmented or depressed by a single compound (dimethylalyl B.A.), depending upon the dose. This reflex can show only depression following the administration of compounds that are convulsive in the intact animal ((1-ethyl-butene-2) ethyl B.A. and 1,3-dimethylbutyl ethyl thio B.A.). Although the spinal flexion reflex is presumably a simpler manifestation of central nervous activity than behavior of the intact animal, its use as in these experiments does not readily permit the drawing of conclusions about the fundamental nature of the action of drugs on motoneurons or at synapses. Concealed inhibitory reflexes have been described (18) for the influence on flexor muscles of stimulation of ipsilateral afferent nerves, and it is conceivable that depression of this mechanism by a drug could result in augmentation of the typical dominant flexion reflex.

Ether and chloroform, which are commonly considered to be depressant drugs, have been shown to have complex influences on spinal reflexes. Sherrington and Sowton (19) showed that contraction of the knee-extensors of the decerebrate cat caused by stimulation of a nerve in the opposite leg was converted to relaxation by the administration of chloroform. Forbes (20) saw a somewhat similar phenomenon in the decerebrate cat, in which ether seemed to have converted reflex excitation into reflex inhibition. Storm van Leeuwen (21) occasionally observed a preliminary augmentation of the flexion reflex in the spinal cat when ether was administered, and Blume (22) saw such augmentation when a solution of ether was injected intravenously. On the administration of ether by inhalation to seven spinal dogs, I have seen only depression of the flexion reflex. In the decerebrate dog, the administration of ether frequently gives a temporary increase in this reflex. This might be interpreted as a release phenomenon, from depression of higher nervous centers, since the flexion reflex is known to be augmented when a decerebrate preparation is made spinal (23). Heinbecker and Bartley (24) have studied the effects of ether and Nembutal on the nervous system, recording the electrical activity in frog, turtle, and cat nerve trunks, the superior cervical ganglion of the turtle, the median nerve cord of *Limulus polyphemus*, and the central nervous systems of the turtle and cat. They concluded that ether first stimulated and then depressed the excitation and response mechanisms, and that Nembutal had a much feebler preliminary stimulating action.

Since the performance of these experiments, Lloyd (25) has shown that the spinal flexion reflex probably involves a three-neurone mechanism at least, while a two-neurone mechanism may be involved with myotatic reflexes (stretch reflex, knee-jerk). The latter, although simpler in mechanism, may also be subject to inhibitory influences, and could show phenomena of "release." This is possibly the explanation of the augmentation, in the whole animal, of the knee-jerk by ethylene and morphine (26). The knee-jerk has been shown to be augmented

by conversion from the decerebrate to the spinal state (31). In the experiments reported here, it was found that the knee-jerk in the anesthetized dog is reduced by small doses, increased by larger doses of two stimulants, strychnine and 1,3-dimethylbutyl ethyl B.A. (See also (26)) It is possible that the smaller doses may have increased the activity of the mechanisms inhibiting the knee-jerk, before augmenting the excitatory mechanism.

It is also difficult to associate the stimulant action of these compounds with any feature of chemical structure. This action is most intense in the case of 1,3-dimethylbutyl ethyl B.A., and completely absent from the effects of most of the closely related compounds studied. Dox and Bywater (27) stated that "Where two branchings occur in the chain, as in 1,2-dimethylpropyl and 1,3-dimethylbutyl, the situation is complicated by a convulsive action of the drug." This is controverted by the purely depressant action of the 1,3-dimethylpentyl and 1,4-dimethylpentyl compounds reported here. The 1,3-dimethylbutyl compound was included in a study by Clowes, Keltch and Krahrl (28) on the influence of barbiturates on *Aibacia* eggs. No divergence in behavior from that seen with the other compounds is apparent in their results.

The introduction of a double bond can confer stimulant properties. 2-methylpropyl methallyl B.A. is depressant (10), while dimethallyl B.A. is stimulant and convulsant. 1-ethyl-butenyl-2 ethyl B.A. gives anesthesia with convulsions in the intact animal, the corresponding butyl compound only depression (8), although both are depressant in the spinal animal. When marked stimulant properties are already present (1,3-dimethylbutyl ethyl B.A.), the introduction of a double bond did not augment the potency.

Benzene derivatives of barbituric acid (position 5) seem to be stimulant only when the substituent group is benzyl, not when it is phenyl or phenethyl, since benzyl ethyl B.A., isopropenyl benzyl B.A. (29), and dibenzyl B.A. (14) are convulsant, phenyl ethyl, phenethyl ethyl, and diphenyl B.A. are not.

Thioarbituric acids are frequently stimulant when the corresponding barbituric acids are depressant. This is apparent with the methallyl compounds, and with phenethyl ethyl B.A. and thio B.A. It has been reported for a large series of compounds by Cope and Hancock (29), and for 5-n-butyl 2-crotyl thio B.A. (30). When the oxygen compound is already highly stimulant (1,3-dimethylbutyl ethyl B.A.), the change for sulfur abolished the stimulant action in the spinal animal, but left it for the intact animal.

Nitrogen substitution seems also to develop or augment stimulant properties. This has been reported by Swanson (15), Tabern and Volwiler (16), for mono-substitution, and by Bush and Butler (17) for di-substitution.

It is of interest that a change in structure that increases the potency of a depressant compound also usually increases the potency of a stimulant compound. Thus 1-methylpropyl and 1-methylbutyl compounds are more active than the propyl and butyl compounds, whether they are the depressant methallyl barbiturates (10) or the stimulant methallyl thioarbiturates. 1-ethyl-butenyl-2 ethyl B.A. and 1-methyl-butenyl-2 B.A. are convulsant anesthetics, the former being more toxic, and the same comparison holds for the 1-ethylbutyl ethyl and

1-methylbutyl ethyl compounds that are depressants (8). 1,3-dimethylbutenyl-1 methyl B.A. is a less potent stimulant than 1,3-dimethylbutenyl-1 ethyl B.A.; depressant barbiturates with a 5-methyl group are generally less potent than corresponding compounds in which this group is ethyl.

SUMMARY. 5,5 (1,3-dimethylbutyl) ethyl barbituric acid produces in intact animals a state of hyperactivity of the central nervous system. Its fatal dose can be raised three times by the previous administration of a depressant barbiturate, but the reverse antagonism is not marked. It augments the respiratory minute volume when this is reduced by barbital or morphine.

This compound augments the flexion and crossed-extension reflexes of decapitate, decerebrate, and anesthetized animals. The knee-jerk of the anesthetized dog is reduced by small doses, increased by larger ones.

All related compounds studied, with substitution of other alkyl groups for the 1,3-dimethylbutyl group, had a purely depressant action on the spinal flexion reflex.

Conversion from barbiturate to thiobarbiturate may develop central nervous stimulant properties, but if these are already apparent in the barbiturate, they may be reduced.

Introduction of a double bond may develop stimulant properties.

The presence of a 5-benzyl group, but not phenyl or phenethyl, favors the presence of stimulant properties.

In several instances, a change in chemical structure which increases the potency of a depressant compound also increases the potency of a stimulant compound.

The spinal flexion reflex may be augmented or reduced by a compound, depending on the dose. Phenomena of central nervous stimulation may be produced in the whole animal by compounds which are purely depressant to the spinal flexion reflex.

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STUDIES ON THE ESTIMATION, ADSORPTION AND PRECIPITATION OF STILBAMIDINE

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One of the most active of the series of diamidines referred to in the previous paper, viz. 4:4'-diamidinostilbene (stilbamidine) shows a strong blue fluorescence in ultra-violet light and this property has been used (1) to study the distribution of the substance in tissues of mice which had received large doses of drug subcutaneously. Examination of the blood of trypanosome-infected mice under a fluorescence microscope showed that the parasites rapidly absorbed the drug after intraperitoneal injection. The fluorescent properties of stilbamidine have also been used for its detection and estimation in biological fluids (2). In this method the fluids are spotted on filter paper and adsorption of the drug occurs as base, thereby providing a very delicate test for the presence of stilbamidine which, however, lacks accuracy as a method of estimation. A colorimetric method for the estimation of the drug in prepared solutions has recently been described (3) and is based on the reaction of glyoxal with the substance in alkaline solution. A similar colour test has also been employed (4, 5) for the estimation of aromatic amidines. None of the methods are specific for the estimation of any member of the series. In order to get further information on the fate of diamidines in the animal body and on the best methods of administration and dosage of these substances, a subject which has already been investigated in trypanosome-infected mice (6) it was necessary to have a satisfactory method for estimating diamidines in biological fluids. In the present investigation this has been accomplished for stilbamidine. The spectrographic method which we have employed in estimating the drug in plasma and serum possesses the great advantage of specificity and should be applicable to those members of the series so far found useful in practice, as all have a definite absorption band in the near ultra-violet (7). Changes in the character of the absorption spectrum after exposure to light whereby the ethylene linkage of stilbamidine becomes saturated, were then readily detected and it is hoped that other changes which may occur as the result of the action of animal tissues may also be observed. The levels of stilbamidine in blood-serum have now been determined in mice after its administration in a single dose by the oral, subcutaneous, intraperitoneal and intravenous routes. The question of the adsorption of the drug by red blood cells and its precipitation by various reagents commonly employed to remove proteins have also been studied.

EXPERIMENTAL. The drug was used as the dihydrochloride in aqueous solution except for intravenous injection when it was dissolved in normal saline. The doses employed for each route were approximately the maximum tolerated

dose for normal mice as well as the minimum curative dose for mice infected with *T. rhodesiense*. The volume of drug solution administered was 0.5 cc. per 20 g. mouse for the oral, subcutaneous and intraperitoneal routes, and 0.2 cc. per 20 g. mouse for the intravenous. The amounts administered per 20 g. by these routes were respectively 8.0, 2.0, 1.0 and 0.2 mgm. (maximum tolerated doses) and 1.0, 0.05, 0.05 and 0.025 mgm. (minimum curative doses). Before oral administration the mice had fasted overnight and the blood was collected at the appropriate time by cardiac puncture after a small flap of skin had been reflected over the sternum. The blood was pooled and allowed to clot and the serum was then obtained by centrifuging. Stilbamidine has a depressant action on the circulatory system and difficulty was sometimes experienced in withdrawing the normal amount of blood while the effect of the drug lasted. The amount of serum used for each estimation was 2.0 cc. but less than this amount would suffice when its drug content is high. At the start we used mice weighing 40–50 g. but later we felt justified in using animals showing greater variation in weight as it has been shown recently (8) that the blood volume of normal animals is proportional to body weight.

It is necessary to remove protein before estimating the drug in serum. For this purpose we tried many precipitants without success as the drug was carried down at the same time. Finally we used dialysed iron as suggested for this purpose (3). The serum was first diluted with two volumes of water, one volume of saline, and the mixture was then heated for 1 minute on the water bath before adding one volume of dialysed iron. The supernatant fluid now diluted to five times its original volume was removed for estimation. Care was taken during the manipulations to exclude light as far as possible since stilbamidine is photolabile in aqueous solution. It was found that the solutions prepared as described showed no loss of drug when kept 24-hours in the dark.

The method of estimation is based on the fact that stilbamidine in aqueous solution exhibits a strong spectral absorption band with maximum at $329\text{ m}\mu$ $\log \epsilon 4.58^1$ (7). The intensity of absorption at $329\text{ m}\mu$ of a suitably prepared serum is compared with that of a pure solution of stilbamidine. The clear solution obtained after protein precipitation from normal serum is never completely transparent at $329\text{ m}\mu$ and this slight absorption has to be considered when the stilbamidine content of serum is estimated. For this purpose the sera of a number of normal mice were used to obtain a correction. Examination of these sera showed an extinction (E , $\log I_0/I$) in a 4 cm. cell of 0.16 ± 0.04 . The value 0.16 is used throughout the present experiments as the "unspecific" absorption of mouse serum treated as above. This value varies with different animal species.

CALCULATION. To estimate the stilbamidine content of serum the fundamental equation $\log I_0/I(E) = \epsilon cd$ is used. A solution of stilbamidine containing $1\text{ }\mu\text{g./cc.}$ will show at $329\text{ m}\mu$ an extinction of 0.45 in a 4 cm. cell. The

¹ The molecular extinction coefficient (ϵ) is defined in the equation $\log I_0/I = \epsilon cd$ where I_0 and I are the intensities of the incident and emergent light, c the concentration of the dissolved substance in gram moles per litre and d the thickness of the absorbing layer in cm.

prepared serum of a mouse 30 minutes after subcutaneous dosage showed an extinction (E) of 0.84 in a 1 cm. cell which is equivalent to 3.36 in a 4 cm. cell. Corrected for unspecific absorption this becomes 3.20, and is due entirely to stilbamidine. The stilbamidine content of the diluted serum is therefore $\frac{3.20}{0.45}$ $\mu\text{g./cc.}$, and five times this amount is in the original serum, viz. 35.5 $\mu\text{g./cc.}$ Numerous preliminary experiments in which known quantities of stilbamidine were added to normal mouse sera showed (Table 1) that the method was capable of measuring the drug content of serum down to 5 $\mu\text{g./cc.}$ within the limits of accuracy of a non-photoelectric spectrographic outfit ($\pm 3.0\%$). Below a drug content of 5 $\mu\text{g./cc.}$ serum the accuracy of the method decreases rapidly due to the preponderance of unspecific absorption, and at levels lower than 2 $\mu\text{g./cc.}$ the method fails to give more than a qualitative picture.

SERUM LEVELS OF DRUG IN MICE. In figure 1 are recorded the serum levels of stilbamidine in mice over the period 0-120 minutes, after a single maximal dose given by the oral, subcutaneous, intraperitoneal and intravenous routes.

TABLE 1
Recovery of Stilbamidine from mouse sera

AMOUNT ADDED IN $\mu\text{g.}$ PER CC. OF SERUM	PERCENTAGE RECOVERED
2.5	90, 110, 140
5	102, 98
10	111, 102
15	103, 104
20	105, 106
25	100, 99
50	96, 99

Estimations of the amount of drug present in sera were actually made up to forty-eight hours after administration but in all cases the amount of drug present after two hours was small (not more than 2 $\mu\text{g./cc.}$). The highest serum level always occurred within thirty minutes after injection, and the peak values did not vary greatly for the different routes in spite of the difference in size of the maximum tolerated doses. It is clear that the drug is rapidly absorbed and that appreciable amounts are found in the blood for relatively short periods only. Following intravenous injection the highest drug concentration was found within a few seconds of administration, and decreased rapidly till after one hour it was too small for estimation. This rapid disappearance of drug from the blood may result from excretion, storage or alteration of stilbamidine in the tissues. It is unlikely, however, that the latter explanation is the true one, for it is stated that up to 50% of the amount of drug administered has been found in the urine (9).

When minimal curative doses of stilbamidine for the four different routes were given to mice as determined in *T. rhodesiense* infections of these animals, only minute amounts of the drug were found in the serum except immediately

after administration by the intravenous route when the value was found to be $15 \mu\text{g./cc.}$ It is clear, therefore, that very low concentrations of the drug are able to destroy the parasites causing this infection. This observation is supported by the fact that stilbamidine given in half the tolerated dose subcutaneously is able to give complete protection to mice against reinoculation with light suspensions of the same parasite for a period of three weeks. The amount

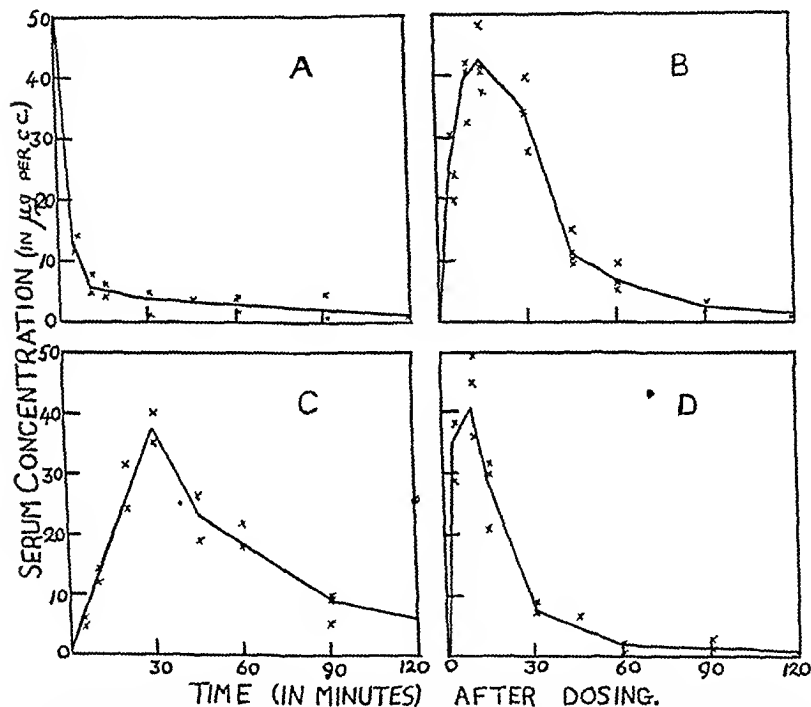


FIG 1. The concentration of stilbamidine in mouse sera during the first two hours after dosing by various routes (a) intravenously, (b) intraperitoneally, (c) subcutaneously, (d) orally.

of drug then circulating must be extremely small. No other explanation of the protection afforded to mice apart from the presence of the drug can readily be given (10).

ADSORPTION OF STILBAMIDINE BY RED BLOOD CELLS. It was shown (2) that stilbamidine is readily adsorbed from solutions by cellulose, animal charcoal, powdered lean beef, etc., and the evidence suggested that red blood cells also adsorbed it. This suggestion has an obvious importance regarding the fate of the drug in the body, especially after intravenous administration. We have, therefore, investigated the matter at some length under the same conditions as described in (2) above.

In table 2 are recorded the results of investigating stilbamidine-adsorption by red blood cells in defibrinated blood of different species. The experiments were all carried out at room temperature. A standard volume of red blood cells was obtained by centrifuging blood samples under identical conditions. The unwashed red blood cells were in contact with serum and the others were washed three times with saline. When the red cells and drug solutions had been in contact for the desired length of time they were separated under the same conditions as originally used and the amount of drug in the supernatant fluid was estimated directly without further treatment. The results show that under the conditions employed adsorption of the drug by red cells occurs, if at all, only to a very limited extent. Any loss shown in our experiments could be explained by dilution of the supernatant by fluid accompanying the red cells.

When red blood cells from citrated sheep's blood (final concentration of citrate 0.5%) were used under the same conditions the amounts of drug recovered at periods of five minutes, two hours and twenty-four hours from washed cells

TABLE 2
Adsorption experiments with Stilbamidine and red blood cells

REACTING SUBSTANCES	TIME OF REACTION	TYPE OF RED BLOOD CELLS USED					
		Human		Rabbit		Sheep	
		Washed	Unwashed	Washed	Unwashed	Washed	Unwashed
		Percentage of drug recovered from solution					
1 cc. red blood cells.	5 min.	91	103	96:102	94:102	90	94
5 cc. of 5 mg. per	1 hr.	90	98	94	94		
100 cc. of Stilbami-	2 hrs.	93	98	94:96	97:94*	89	94
dine in saline	24 hrs.	93	97	101	101	90	89

were 94, 96 and 90%, and were 100, 100 and 87% from unwashed cells. In order to approach more natural conditions the following experiment was carried out with defibrinated sheep and rabbit blood. 1 cc. samples of unwashed cells were mixed with 2 cc. serum and 2 cc. of 5 mgm./100 cc. of stilbamidine in saline. The mixtures were allowed to stand for two hours and twenty-four hours, respectively, with occasional shaking and the amount of stilbamidine in the supernatant fluid estimated after precipitation of protein. The amount recovered in both cases was between 90 and 100%. In control experiments the drug was mixed with the respective sera in the absence of red cells and estimated at the same times as above; the amount recovered after two and twenty-four hours was again between 90 and 100%. We also carried out a number of experiments in which the blood from each mouse in a batch was divided into two portions. The amount of drug in the serum was then estimated in one portion as soon as clotting had occurred and in the other portion after the serum had stood in contact with the clot overnight. There was no appreciable difference in the amount of drug present in either case. From these experiments we conclude that adsorption of stilbamidine by red cells does not occur to any

appreciable extent. This result is in complete contrast to those previously recorded (2).

PRECIPITATION REACTIONS WITH STILBAMIDINE. During the course of our work on the estimation of stilbamidine we found that it was readily removed from solution by a number of protein precipitants, a fact to which attention has already been drawn (3). These precipitation reactions have been investigated in some detail on account of their practical importance. The results are shown in table 3.

The highest concentration of stilbamidine used was 2 mgm. per cc. in water and saline, since this is the strength commonly employed in human treatment. It will be noted that precipitation occurs over a wider range of concentrations

TABLE 3
Precipitation reactions with Stilbamidine

REAGENT	MGm. STILBAMIDINE IN 1 CC. SOLUTION					
	20	10	0.5	0.25	0.1	0.05
Human serum	+	+	±	0	0	0
Rabbit serum	+	0	0	0	0	0
Mouse serum	+	+	±	±	0	0
Sheep serum	±	0	0	0	0	0
2.5% sod citrate	+	+	+	+	0	0
0.5% sod citrate	+	+	+	+	0	0
Trichloroacetic acid 10% and 100%	+	+	+	+	+	+
Perchloric acid 20%	+	+	+	+	0	0
Metaphosphoric acid 20%	+	+	+	+	+	+
10 N/HCl	+	+	+	+	±	0
N/HCl	0	0	0	0	0	0
Absolute alcohol	0	0	0	0	0	0

+

 = Immediate precipitate.

±

 = Appearance of precipitate delayed

0

 = No precipitate

with human and mouse sera than is the case with those of rabbit or sheep. With sheep serum, however, precipitation is marked with drug strengths of 4 mgm. per cc. or over, a point to which we shall refer later. Water or saline in absence of drug does not give rise to precipitation with these sera.

The reaction was investigated quantitatively in the case of human serum mixed with an equal volume of 2 mgm. per cc. of stilbamidine solution. It was found that the precipitate consisted of protein and what we believe to be stilbamidine base since unlike the dihydrochloride used it was insoluble in water but soluble in dilute HCl. The precipitate contained 2.7% of the amount of drug added while the centrifuged supernatant contained 87.5%, accounting for 90.2%. Estimation of chloride ion in the supernatant was not of much help owing to the presence of large quantities already in the serum. When equal volumes of 0.5% sodium citrate and stilbamidine solution (2 mgm. per cc.)

were mixed the precipitate was found to contain 94% of the added drug and the supernatant 3.7%. We are unable to account for the statement (2) that citrate causes no precipitation with stilbamidine. The crystalline precipitate is almost certainly the citrate; it was only slightly soluble in water, readily soluble in dilute HCl, and the amount of chloride ion in the supernatant was not altered after precipitation. According to Dr. A. J. Ewins, who has a wide experience with diamidines, most salts of the same type are very insoluble. Trichloroacetic and metaphosphoric acids precipitate the drug from its solution in very low concentrations and cannot be used as protein precipitants in estimating the drug. Perchloric acid, recently introduced as a deproteinising agent (11), may possibly find application in this connection. Normal or basic lead acetate in saturated solution does not cause precipitation of the drug.

DISCUSSION. A spectrophotometric method has been found suitable for estimating the amount of stilbamidine in the sera of treated mice. Estimations have been carried out over periods of forty-eight hours after administration by four different routes. The doses used were the maximum tolerated, and the minimum curative for *T. rhodesiense* infections in mice. After the latter dose only minute amounts of drug were present in the serum. The peak levels for the maximum tolerated doses were found to occur in each case within thirty minutes after administration, and were followed by a rapid diminution in the amounts present which after two hours were too small for estimation. The levels attained by all four routes were approximately the same and support the view previously expressed (6), as the result of therapeutic tests in *T. rhodesiense* infections in mice, that the curative indices are largely conditioned by the amount of drug tolerated when given by the respective routes. After subcutaneous administration the somewhat slower fall in the serum content of drug probably also contributes to the high value of the curative index. Whether the rapid disappearance of the drug from the sera is due to excretion, storage or degradation in the tissues has not yet been determined.

It has been suggested (2) that 60-80% of the stilbamidine present is adsorbed from solution by red blood cells suspended in it. We have been unable to obtain evidence that adsorption by red cells occurs to any appreciable extent, either after washing them with saline or when they are left in contact with serum. In the original experiments citrated blood was sometimes used and the statement made that citrate does not precipitate stilbamidine from solution. We on the other hand found that precipitation is almost quantitative by this reagent at certain concentrations in confirmation of previous findings (3). Another source of error in the experiments under discussion was the addition of a concentrated solution of stilbamidine (strength not stated) to a suspension containing sheep serum. We have drawn attention to the precipitation of drug which occurs under these conditions. Moreover, these workers did not recover stilbamidine from red cells even after haemolysis, which is not surprising since if the drug were adsorbed as base (as occurs on filter paper) its insolubility in the organic solvents used would prevent ready extraction. Sulphuric acid (1%) was also used for extraction purposes. Stilbamidine sulphate is, however,

so insoluble that this property makes the estimation of the drug as sulphate feasible when precipitated from solution by sulphuric acid.

Our experiments dealing with the precipitation of stilbamidine from solution by various reagents indicate the difficulties which are encountered in obtaining protein-free filtrates from biological solutions containing the drug.

SUMMARY

1. A spectrophotometric method has been described for the estimation of stilbamidine in serum.

2. The drug-levels in the sera of mice treated by the oral, subcutaneous, intraperitoneal and intravenous routes were estimated at intervals over periods of forty-eight hours. The drug was rapidly absorbed, the peak values occurring in all cases within 30 minutes after administration. At the end of two hours only minute amounts were present.

3. No unequivocal evidence was obtained for the adsorption of the drug from solution by suspensions of red blood cells.

4. A series of precipitation reactions with solutions of the drug and various reagents has been described.

One of us (J. D. F.) is a member of the scientific staff of the Medical Research Council.

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THE EFFECT OF LIGHT ON VARIOUS AROMATIC DIAMIDINES IN THE SOLID STATE

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Some aromatic diamidines prepared by Dr. A. J. Ewins, have been found very active against various protozoal diseases including trypanosomiasis, kala-azar and piroplasmiasis.

In Gambia (1) it was first noticed clinically that solutions of 4:4'-diamidino stilbene (stilbamidine) increased in toxicity some hours after preparation: no reason for this increased toxicity was given. Later (2), in the Sudan, various toxic symptoms were noted in cases of leishmaniasis treated with the same drug. It was then shown experimentally (3) that solutions of stilbamidine which have been exposed to light become more toxic for mice. Concurrently the therapeutic activity of the drug diminishes and this fact has been repeatedly confirmed. It has been suggested from experimental evidence (4) that this change was photochemical in character, involving the addition of water to the ethylenic linkage with formation of 4:4'-diamidinophenylbenzylcarbinol. An alternative suggestion (5) is that 1:2:3:4-tetra-(4'amidinophenyl)-cyclo-butane is formed from two molecules of stilbamidine. Other aromatic diamidines with acetylenic or ethylenic linkages also undergo photochemical changes in aqueous solution (6), and the changes in the absorption spectra of these solutions indicate that the saturation of the double (or triple) bond is the main change taking place (7). These photochemical changes are obviously of great importance in the tropics and the clinical aspects of the subject have recently been investigated (8).

It seemed to us of importance for both theoretical and practical reasons to investigate the change undergone by the solid substances when acted on by light, especially as both solutions and solids become discoloured by its action. This communication records the results of these investigations on three diamidines by biological and spectrographic methods.

EXPERIMENTAL. The dihydrochlorides of 4:4'-diamidinostilbene, 4:4'-diamidinotolane and 4:4'-diamidino- α -monomethylstilbene, all of which are known to undergo toxic changes with partial loss of therapeutic activity when exposed to light in aqueous solution, were exposed in the solid state to light and air under glass in the laboratory for periods of 8-14 months. Samples were taken at intervals and examined biologically for toxicity in mice as well as spectrographically for any change in either the position or intensities of absorption bands.

The first two substances were colourless at the start while the last had a faint yellow tinge. After one week's exposure all were definitely coloured, and

finally stilbamidine became bright yellow while the other two substances became yellowish-brown.

It has been pointed out by several authors that solutions of stilbamidine, as well as the solid, became yellow due to the action of light. The solid, however, does not show the phenomenon of phototropy which is exhibited by certain stilbene derivatives; nor do its solutions, if the same term may be applied. We have found that once the colour change has taken place there is no reversion to the colourless state after several months in the dark.

TABLE 1

Showing toxicity for mice of solutions of unexposed and exposed diamidines

		DOSE IN MCM. PER 20 GRAM MOUSE					
		2.0		1.0		0.5	
4:4'-Diamidino-stilbene	Unexposed	10	2D 1P 7S	10	1P 9S	5	5S
	Exposed 14 months	10	2P 8S	10	10S	9	9S
4:4'-Diamidino-tolane	Unexposed			10	8D 2S	10	1P 9S
	Exposed 8 months			10	10P	10	10S
4:4'-Diamidino- α -mono-methyl-stilbene	Unexposed	15	10P 5S	10	10S	5	5S
	Exposed 8 months	10	8D 2S	10	2D 8S	5	5S

D = Died within an hour after injection.

P = Died within a few days after injection.

S = Survived for 21 days after injection.

At the end of 8 months exposure solutions were made of each substance and their absorption spectra compared with those of similar solutions of the unexposed material. No significant change had taken place in the spectra of any of the compounds and no new absorption bands were present. Using a colorimetric method (9) the loss of stilbamidine which had become yellow after a few days exposure was estimated to be about 5% of the original, while it has also been found (8) that yellow stilbamidine is not more toxic for mice after 14 days exposure.

In the present experiments the yellow stilbamidine resulting from 14 months exposure showed a loss of only 5-8% in different samples as determined spectrographically. The above results suggest that the change occurs within a few days and is probably only a surface change. The results of toxicity investigations in mice with three diamidines are shown in table 1.

These results suggest that there has been no significant change in toxicity following exposure, and that the maximum tolerated dose of each substance is unaltered. In the case of stilbamidine the therapeutic activities of the fresh and exposed substances were tested over a critical range in *T. rhodesiense* infections of mice with the results shown in table 2.

These tests indicate that in the case of the two lowest doses the therapeutic activity of the exposed substance has been reduced; a fact which is in agreement with the 5-8% decrease in intensity of absorption shown spectrographically.

TABLE 2

Therapeutic experiments on infected mice with unexposed and exposed stilbamidine

	DOSE IN MG. PER 20 GRAM MOUSE					
	0.025		0.01		0.005	
Unexposed material.....	10	10C	15	7R 8C	15	3N 8R 4C
Exposed material.....	15	1R 14C	15	10R 5C	15	10N 2R 2C

C = Cured.

R = Blood became negative but relapse occurred.

N = Blood never free from parasites.

TABLE 3

Toxicities of yellow and colourless stilbamidine after exposure in solution

	DOSE IN MG. PER 20 GRAM MOUSE					
	1.5		0.25		0.1	
Colourless stilbamidine exposed 2 days.....	3	3D	3	1D 2S*	3	3S
Yellow stilbamidine exposed 2 days.....	3	3D	3	3D	3	3S

D = died in less than 1 hr.

S = survived for 21 days.

* Animals survived but were very toxic after injection.

In order to find out if the yellow stilbamidine underwent the same toxic changes in aqueous solution as the fresh material, 1% solutions of each were exposed for 2 days in sunlight and their toxicities tested in mice by giving 0.5 cc. of a solution of the desired strength intraperitoneally to each 20 gram mouse. It will be seen from table 3 that the yellow stilbamidine undergoes the same toxic changes as the unexposed material when both are exposed in solution for two days. This result is in agreement with the view already expressed that only a surface alteration has occurred.

SUMMARY

1. Biological and spectroscopic investigations indicate that the colour change occurring in 4:4'-diamidinostilbene, 4:4'-diamidinotolane and 4:4'-diamidino- α -methylstilbene following exposure to sun and air in the solid state for periods of 8-14 months is not accompanied by significant changes either in toxicity or in absorption spectra.

2. In the case of stilbamidine slight loss of therapeutic activity was noted after 14 months only with minimal doses.

3. Stilbamidine which has become yellow from exposure undergoes the same increase in toxicity in solution as the unexposed solid.

4. The dihydrochlorides of the aromatic diamidines investigated may safely be used clinically after long exposure to air and sunlight as solids.

One of us (J. D. F.) is a member of the scientific staff of the Medical Research Council.

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ADDITIONAL STEROIDS WITH LUTEOID ACTIVITY

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It has been assumed, mainly on the basis of Butenandt's (1) work, that luteoid activity is a specific property of progesterone. This author stated that an α , β -unsaturated ketone group at C_3 is indispensable for luteoid activity and indeed that any change in the molecule of progesterone results in its complete inactivation. The work of Klein and Parkes (2) showed, however, that these rules are not valid since compounds, such as testosterone and methyl-androstenediol, are likewise capable of producing progestational proliferation of the endometrium. On the basis of systematic bioassays with 45 steroid compounds we were able to detect certain correlations between chemical structure and the luteoid activity (3, 4). The present communication represents a direct continuation of this work and contains data concerning the progestational activity of additional steroids.

EXPERIMENTAL. All compounds were uniformly assayed on immature female rabbits according to the technic of McPhail (5). In order to make all our observations comparable, the experimental procedure was exactly identical with that used in our previous work (4).

Table 1 summarizes our observations. In the first column the steroids are arranged in the order, and designated by the full systematic chemical names recommended by the Encyclopedia of Endocrinology (6); the common names are also mentioned (in italics) wherever such are in general use. The names of the chemists who supplied us with these compounds are listed in brackets. The second column gives the melting point of the sample we used and, in brackets, the melting point of pure samples as recorded in the literature; this helps to estimate the degree of purity of the samples employed. The third, fourth and fifth columns register, respectively, the total dose administered per animal, the number of animals used and the endometrial response obtained at each dose level. The degree of progestational proliferation was estimated according to the McPhail scale and is expressed as ranging from 0 to + + + +. Whenever several animals responded differently at the same dose level, the numeral in brackets refers to the number of animals which gave an identical response. One rabbit unit in this test represents the amount necessary to produce a response of + + to + + + and is equal to 0.25-0.5 mg. of progesterone.

RESULTS. The present experimental series confirms some of our previously described observations concerning the correlations between chemical structure and luteoid activity. Derivatives of the etiocholane series are inactive (cpds. 15, 16, 17). Complete saturation of the molecule is compatible with luteoid activity (cpd. 7). At position C_{17} a β -hydroxyl is less favorable for luteoid activity than an α -hydroxyl (compare cpd. 8 or "cis-testosterone," with our

previously published observations concerning common testosterone). It should be kept in mind also that, in general, esterification with acetic or propionic acid increases the luteoid activity of such compounds (e.g. that of common or 17(α)-testosterone), while in the present experimental series even the acetate and the propionate of cis-testosterone proved to have no definitely positive luteoid activity. From this it may be concluded that the cis-testosterone molecule is devoid of any appreciable inherent luteoid potency irrespective of the form in which it is administered. Among the steroids possessing a ketone group at C₁₇ (cpds. 5, 6, 7) the simultaneous presence of a second ketonic oxygen at C₃ is more favorable for luteoid activity than either a free α - or a free β -hydroxyl in this same position.

It is of interest to note that none of the i-androstane derivatives, tested in the present series, proved to have any luteoid activity (cpds. 1, 2, 3). It must be kept in mind, however, that those which possess a long side chain at C₁₇ (cpds. 2, 3) are necessarily inactive by virtue of this fact, since among all steroids examined up to the present time, none proved to have luteoid activity if their molecule possessed a side chain of more than four carbon atoms at C₁₇. With regard to i-pregnenolone, we wish to emphasize, however, that owing to lack of material this compound was assayed on one rabbit only and at a comparatively low dose level of 14 mg. The corresponding Δ^5 -pregnenolone gives but a slight response at a 10 mg. dose level (4) and since methyl ethers are generally less active than the corresponding free compounds, the observed inactivity of the i-pregnenolone methyl ether cannot be regarded as proof that the i-androstanes are less likely to possess luteoid activity than the corresponding Δ^5 -androstenes. Further i-androstane derivatives are being investigated in this laboratory with the view of clarifying this point.

If we compare non-alkyl-substituted steroids with those possessing a methyl, ethyl or ethynyl group at C₁₇ (see reference 4), the following conclusions can be drawn: addition of a methyl, ethyl or ethynyl group at C₁₇ increases the activity of compounds which normally possess but slight luteoid properties (e.g. Δ^5 -ANDROSTENE-3(β),17(α)-diol, testosterone, ANDROSTANE-3(β),17(α)-diol). Klein and Parkes (2) noted that "methyl-androstanediol (cis)" and "ethyl-androstanediol (cis)" give a + to ++ response at a dose level of 10 mg., while "androstanediol" is inactive at the dose of 20 mg.; however, owing to the ambiguity of the terminology used, the true identity of the above mentioned compounds cannot be established. Lack of material prevented us from assaying methyl-ANDROSTANE-3(β),17(α)-diol and methyl-ANDROSTANE-3-one-17(α)-ol at any dose level lower than 12.5 mg. Since the relationship between dose and response is not always strictly quantitative, it is quite possible that smaller amounts of these steroids would still prove to be effective.

We were particularly interested in the fact that ethynyl-androstenediol proved to be a very potent luteoid compound. Since this steroid is an intermediary product in the synthesis of ethynyl-testosterone, the practical significance of this finding is obvious. In order to evaluate this new luteoid, we decided to examine its luteoid, testoid and folliculoid activities comparing them with those of ethynyl-testosterone.

TABLE I
Luteoid activity of steroid hormones

NO	COMPOUND	M.P. °C	DOSES ANIMALS	NO OF ANIMALS	RESPONSE
1	17(α) [1 ketoethyl] 1 ANDROSTANE 6(?) ol methyl ether, 1-pregnalone methyl ether (Pike)	121-5 (121-5)	14	1	0
2	17(α) [1(?) carboxyethyl] 1 ANDROSTANE 6(?) ol methyl ether methyl ester, 6 methoxy 1-bisnor cholanate methyl ester (Pike)	glassy material	50	3	0
3	17(α) [1,5 dimethylhexyl] 1-ANDROSTANE 6(?) ol methyl ether, 1-cholesteryl methyl ether (Pike)	79	50	3	0
4	ANDROSTANE 3(β), 17(α)-diol (Scholz)	163-4 (168)	50	3	0(1) + (2)
5	ANDROSTANE 3(α) ol-17-one, <i>cis</i> androsterone (Hoffman)	178-80 (180-1)	50	1	0
6	ANDROSTANE 3(β) ol 17 one, <i>iso</i> androsterone (Hoffman)	165-8 (176-7)	50	2	0(1) \pm (1)
7	ANDROSTANE 3, 17-dione (Heard)	132-4 (133-4)	50	1	++ to +++
8	Δ^4 ANDROSTENE 3 one-17(β)-ol, <i>cis</i> testosterone free compound (Holden), acetate (Holden), propionate (Holden)	215-7 (220-1) 113-4 (115 5-6 5) 92 5-3 5 (92 5-3 5)	50 50 50	3 4 3	0(2) \pm (1) 0(2) \pm (2) 0(2) \pm (1)
9	17(β) methyl-ANDROSTANE-3(β), 17(α)-diol (Scholz)	211-3 (211-2)	50 25 12 5	3 2 3	++(2) + + + (1) ++(2) + + + (1) ++

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		188 (192-3)			
10	17(β)-methyl-ANDROSTANE-3-one-17(α)-ol; methyl-dihydrotestosterone (Holden)	50 25 12.5	3 3 2		++(1)+++ (2) +(1)++(1)+++ (1) ++(1)+++ (1)
11	17(β)-methyl- Δ^4 -ANDROSTENE-3(β), 17(α)-diol; methyl-androstenediol (Holden)	204.5 (204)			++(1)+++ (1)+++ (1) ++(1)+++ (1) ++(1)+++ (1) ++(2)+++ (2) +(3)++ (1)
12	17(β)-ethyl-ANDROSTANE-3-one-17(α)-ol; ethyl- dihydrotestosterone (Holden)	131-8 (137-8)	50 35 12.5 7.5 5	3 2 3 3 4	++(1)+++ (1)+++ (1) ++(1)+++ (1) ++(1)+++ (1) ++(2)+++ (2) +(3)++ (1)
13	17(β)-ethyl- Δ^4 -ANDROSTENE-3-one-17(α)-ol; ethyl-testosterone (Pike)		12.5 7.5 5	3 3 3	++(1)+++ (1)+++ (1) +(1) to ++(1)++ to +++ (1) 0 to +(1)++ (2)
14	17(β)-ethynyl- Δ^4 -ANDROSTENE-3(β), 17(α)-diol; ethynyl-androstenediol (Pike)	138-9.5 (139)	12.5 7.5 5	3 3 3	++ to ++(1)+++ (2) ++(1)++ to ++(2) 0 to +(1) to ++(1)++ (1)
15	17(α)-[1-ketoethyl]-ETIOCHOLANE-3, 12-dione; pregnanetrone (Pike)	230-42 (243-5)	50 25 12.5 7.5 5	1 2 2 3 5	++(1)+++ (1) ++(1)++ to ++(1) ++ to +++ + to ++(1)+++ (1)+++ to +++ (1)
16	17(α)-[1(γ)-carboxyethyl]-ETIOCHOLANE-3(α), 12(β)-diol; bismor-desoxycholeic acid (Heard)	194-7 (201-2)	50	1	0
17	17(α)-[1(γ)-methyl-2-carboxyethyl]- ETIOCHOLANE-3(α), 12(β)-diol; nor-desoxy- choleic acid (Heard)	213 (195-202)	50	3	0
		201.5-2.5 (211-2)	50-	3	0

The luteoid potency of these substances, given either by mouth or subcutaneously, was again tested according to McPhail's method. For oral administration the total amount to be given was dissolved in 10 cc. of oil and 2 cc. of this solution were administered daily through a stomach tube. Table 2 indicates that ethynyl-androstenediol is as potent *per os* as parenterally and given either way it proves to be only slightly less active than ethynyl-testosterone.

The testoid assays were performed on male albino rats weighing 40-60 g. (average 50 g.). The animals were divided into four groups of 10: two groups received respectively 1 and 2 mg. of ethynyl-androstenediol subcutaneously daily and the other two groups were similarly treated with ethynyl-testosterone.

TABLE 2
Comparative luteoid assays with ethynyl-androstenediol and ethynyl-testosterone

	DOSE	RESPONSE	
		Ethynyl-testosterone	Ethynyl-androstenediol
	mg.		
a) Subcutaneous administration	5	±(1) ++(3) ++ to +++(1)	+ to ++(3) ++(2)
b) Oral administration	10	+++ (2) +++ to ++++ (1)	++(1) ++ to +++(2)
	5	+(1) ++ to +++(1)	+(1) + to ++(2)

TABLE 3
Comparative testoid assays with ethynyl-androstenediol and ethynyl-testosterone

NAME OF COMPOUNDS	DAILY DOSE	SEMINAL VESICLES	PROSTATE
	mg.	mg.	mg.
Ethynyl-androstenediol	1	17 (10-25)	41 (33-48)
	2	21 (18-26)	50 (38-62)
Ethynyl-testosterone	1	33 (27-44)	64 (54-83)
	2	48 (36-60)	69 (46-92)

Treatment commenced 24 hours after castration and continued during 10 days. The animals were sacrificed on the 11th day; the accessory sex organs were removed, fixed in "Suza" and subsequently weighed. The results are summarized in Table 3. It will be noted that the seminal vesicles and prostate are definitely less stimulated by ethynyl-androstenediol than by corresponding amounts of ethynyl-testosterone. These results could be expected by analogy with other compounds, thus testosterone stimulates the accessory sex organs more intensely than Δ^5 -ANDROSTENE-3(β),17(α)-diol (7). It will be recalled that for clinical use the main disadvantage of ethynyl-testosterone as a luteoid compound is its comparatively strong associated testoid activity; hence, the

less virilizing ethynyl-androstenediol would appear to be preferable in gynecological practice if its luteoid potency proved to be satisfactory.

The folliculoid assays were performed on spayed, immature albino rats, which were divided into two groups of 10 animals each. After preliminary assays we decided to inject ethynyl-androstenediol at a dose of 25 γ and ethynyl-testosterone at a dose of 50 γ twice daily subcutaneously in cotton seed oil. Treatment was continued for a period of 10 days during which time the vagina was examined to determine whether opening took place. Smears were taken daily in rats in which the vagina opened. On the 11th day the animals were killed and the accessory sex organs (fixed in "Suza") weighed and histologically examined. The results may be summarized as follows: In the group receiving ethynyl-androstenediol the vagina opened on the 4th day and the smears contained only nucleated or cornified cells; the increase in uterine weight amounted to 140%. In the group receiving ethynyl-testosterone, the vagina remained closed throughout the treatment and the increase in uterine weight was only 50%. On histologic examination the vagina was found to be atrophic or mucified, but not cornified. Thus ethynyl-androstenediol proved to be more folliculoid than ethynyl-testosterone. In this connection it will be recalled that Δ^5 -ANDROSTENE-3(β),17(α)-diol is more folliculoid than testosterone (8) so that our observations with the corresponding C_{17} ethynyl derivatives could be expected by analogy.

On the basis of the above mentioned observations it may be said that ethynyl-androstenediol is only slightly less luteoid, but considerably less testoid and more folliculoid than ethynyl-testosterone. It may also be stated that the addition to a testoid compound of a C_{17} alkyl group decreases its testoid activity, but increases its luteoid and folliculoid activity and that C_{17} ethynyl derivatives are as active when given orally as they are subcutaneously.

SUMMARY

17 steroid compounds have been studied for luteoid activity. In agreement with some of the already known pharmaco-chemical correlations, it is concluded that the addition of a methyl, ethyl or ethynyl group at C_{17} markedly increases the progestational effect of parent compounds which possess only a slight degree of luteoid activity. This general rule has now been demonstrated for testosterone, dihydro-testosterone, Δ^5 -ANDROSTENE-3(β),17(α)-diol and ANDROSTANE-3(β),17(α)-diol.

Comparative studies concerning three of the main pharmacologic activities of ethynyl-androstenediol and ethynyl-testosterone are reported. These indicate that ethynyl-androstenediol is only slightly less luteoid than ethynyl-testosterone, but considerably less testoid and more folliculoid than the latter compound. The practical significance of these observations is discussed.

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THE TOXICOLOGY OF 1,2-DICHLOROETHANE (ETHYLENE)

III. ITS ACUTE TOXICITY AND THE EFFECT OF PROTECTIVE AGENTS

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1,2-Dichloroethane ($\text{CH}_2\text{Cl}-\text{CH}_2\text{Cl}$) is a chlorinated hydrocarbon which became of wartime industrial importance as an intermediate in the manufacture of thiokol synthetic rubber. The compound is widely used as a solvent and as an insecticidal fumigant. It is a colorless liquid with an odor somewhat resembling chloroform. It has a specific gravity of $1.257 \frac{20^\circ\text{C.}}{4}$, a boiling point of 83.7°C. and an index of refraction, $n_{20^\circ\text{C.}}$, of 1.44432 (1). Dichloroethane is miscible with the ordinary organic solvents and dissolves in water to the extent of 0.869 gms. per 100 cc. at 20°C.

The toxic properties of this compound are of interest because of the fact that industrial exposures to the vapors take place. Comparatively few pharmacological studies are recorded in the literature. Its narcotic action is said to be of the same order as that of chloroform. Lehmann and Schmidt-Kehl (2) carried out studies on cats and rabbits and found dichloroethane to be more potent than ethyl chloride. The concentrations causing light and deep narcosis were very close together. Kistler and Luckhardt (3) anesthetized dogs with dichloroethane and found that after 2 minutes of marked salivation and excitement the animals became deeply narcotized. Recovery was rapid. Dogs treated with the intravenous injection of 0.25 cc./kg. of dichloroethane died whereas 0.125 cc./kg. did not cause depression or serious after-effects. The strong clouding of the cornea of dogs which is caused by this solvent has been studied by several investigators. The pertinent literature and observations made in this Laboratory are considered elsewhere (4).

Two investigators have reported that mice dying from the effects of single inhalation exposures to dichloroethane showed fatty degeneration of heart, liver and kidneys (5, 6). Sayers, Yant, Waite and Patty (7) exposed guinea pigs to various concentrations of dichloroethane. They found that 6 volume per cent caused irritation of the eyes, vertigo, static and motor ataxia, twitching movements, semiconsciousness and death in 30 minutes. A concentration of 0.12 volume per cent caused no apparent symptoms, but the animals died 8 hours

after the exposure. Pathological effects were congestion and edema of the lung and renal degeneration.

In the present investigation studies have been carried out on the acute toxicity of dichloroethane when administered orally, by subcutaneous injection, by intraperitoneal injection and by inhalation. Eight different species of animals were tested for the inhalation work. The action of various protective agents was studied in mice. It was found that methionine, p-amino benzoic acid, sulfanilamide and aniline protected mice against the lethal effects of dichloroethane poisoning.

EXPERIMENTAL. The dichloroethane was a commercial product of high purity as judged by its specific gravity, index of refraction and the results of precision distillation (6 foot fractionating column). The material was also checked for the absence of aldehydes chloride, free acid, water and substances decomposed by sulphuric acid. The results of tests on the several lots will be published in the next paper of this series.

The inhalation exposures were carried out in a chamber measuring 4' x 4' x 6', which permitted reasonable numbers of animals of different species to be exposed simultaneously. Air was drawn through the chamber by means of a vacuum pump at a rate sufficient to renew the air every 7 minutes. Dichloroethane was volatilized at a constant rate by passing a steady flow of air through the liquid. The vapors joined the main air stream entering the chamber. Details of construction and operation are given elsewhere (8). The concentration of dichloroethane in air which was established could be calculated from the rate of volatilization and the total air flow through the chamber. The values were checked by daily chemical analyses of gas samples taken from the chamber. Dudley's method was used for the determination (9). As a further control procedure, measurements were made at hourly intervals with the Raleigh-Jeans interference refractometer. The temperature and relative humidity inside the chamber were measured daily. Both were moderate during the seasons when the work was done.

For subcutaneous injection the dichloroethane was dissolved in olive oil, while for intraperitoneal inoculation the material was dissolved in 0.9 per cent sodium chloride solution.

Diets. The dogs were fed equal parts by weight of ground horse meat and Kippell biscuits. Rats and mice received Purina dog checkers supplemented with greens. Guinea pigs and rabbits received greens, carrots and pressed pellets whose composition is given elsewhere (6). The monkeys received Chim crackers, whole-wheat bread, milk, eggs and a variety of fruits and vegetables.

The rats were of Wistar strain. Mice of various strains from the National Cancer Institute were used.

The animals were observed both day and night after an acute exposure and all autopsies were performed soon after death. Sections of liver, heart, lungs, kidney, adrenal glands and spleen were taken routinely for microscopic examination. Femurs of some rats were examined. Sections of brain and spinal cord were prepared for rats and dogs. Tissues were fixed in 3.7% aqueous formaldehyde, embedded in paraffin, sectioned, and stained with eosin-azure and with iron-hematoxylin-picricfuchsin. Frozen sections of liver and kidney were stained for fat by the method of Lillie and Ashburn. (10). Sections of nervous tissue were stained by the Marchi and Weil methods for myelin, and with eosin-azure.

RESULT. *Part 1. Inhalation exposures to 12.4 mg./liter* (approximately 3,000 parts per million).

In table 1 are shown the results of exposing 7 species of animals to 3000 parts per million (p.p.m.) of dichloroethane.

Sixteen rabbits were exposed for 7 hours. All but 4 succumbed. Death

took place in 5 to 72 hours after the exposure period ended. In the chamber some of the rabbits appeared mildly narcotized while others were prostrate. There was no evidence of local irritation. After removal to their cages they recovered and appeared almost normal for a time. Then their eyes became sunken, they developed increasing dyspnea and fell on their sides. Gross autopsy revealed small amounts of peritoneal fluid in about one-fourth of the animals. Several showed small amounts of fluid in the chest. All of the animals had pulmonary involvement which varied from mild congestion to actual scattered hemorrhages. Congestion was noted in the abdominal viscera. Microscopically

TABLE 1
Mortality after single exposures to dichloroethane

ANIMALS	NUMBER	AVERAGE WEIGHT	LENGTH OF EXPOSURE	MORTALITY	TOTAL NUMBER OF DEATHS					
					At end of exposure	Days after exposure				
						1	2	3	4	5
3,000 p p m										
		grams	hours							
Rabbits	16	3,940	7	12/16	0	7	11	12		
Guinea pigs	14	885	7	14/14	0	11	13	14		
Hogs	2	27,300	7	2/2	0	0	2			
Cats	3	3,240	7	0/3						
Raccoons	2		7	0/2						
Mice	22		7	22/22	22					
Mice	19		2	19/19	0	19				
Rats	20	146	7	20/20	0	19	20			
Rats	16	177	3½	15/16	0	1	3	5	13	15
Rats	15	257	1½	0/15						
1,500 p p m.										
Guinea pigs	12	321	7	6/12	0	1	4	5	6	
Mice	20		7	20/20	4	20				
Mice	23		2	1/23	0	0	0	1		
Rats	20	170	7	4/20	0	2	2	4		
Rats	13	257	4	0/13						

(6 rabbits), there was generalized visceral congestion and slight to moderate hepatic necrosis. The architecture of the liver was not disrupted and the degenerating and necrotic liver cells were irregularly distributed in the cords. Each rabbit showed fatty degeneration of renal tubular epithelium of slight degree.

Fourteen guinea pigs were exposed for 7 hours to 3000 p.p.m. of dichloroethane, which killed all of them within 3 days. During exposure the pigs developed an uncertain gait, considerable lacrimation, and moisture about the mouth. After exposure the animals were quite inactive and breathing became labored. An occasional pig showed mucopurulent material coming from its nostrils. Positive findings on gross autopsy were limited largely to the lungs, with varying

degrees of congestion and, in some of the pigs, the occurrence of clear pleural fluid. Microscopic examination of 11 pigs showed visceral congestion in all of them. Liver, lung and adrenal gland were particularly affected. Focal necrosis of the adrenal cortex was seen in 5 pigs and this was associated with hemorrhage in 3 of them. Slight to moderate fatty degeneration of renal tubular epithelium was noted in 8 animals.

On another occasion 8 guinea pigs of comparable size were similarly exposed. All but one were dead by the next day. Microscopic study of tissues from 7 pigs showed fatty infiltration of the myocardium in all and pulmonary congestion in 5 of them. Three showed fatty infiltration of convoluted and collected renal tubules. One showed congestion and necrosis of the adrenal cortex.

Mice which died after exposure showed nothing grossly except pulmonary congestion. Microscopic examination of the tissues of 8 mice showed generalized visceral congestion in all and slight fatty degeneration of the liver in 6. The hepatic lesions were patchy and without any recognizable tendency to affect any particular part of the lobule.

Twenty young adult rats, mostly males, showed responses to the 7-hour exposure which varied from mild narcosis to complete loss of consciousness. The rats were alive at the end of the exposure period but all died during the next 48 hours. Grossly, the lungs were moderately congested and there was occasional pleural and abdominal fluid.

Tissues of 10 rats were examined microscopically. All showed some congestion of the viscera, particularly of liver and spleen. In 8 of the 10 there were scattered foci of degeneration and necrosis in the liver and in 9 there was slight to moderate fatty degeneration of this organ. In general the fatty degeneration was most prominent in portal areas. Unfortunately sections of adrenal gland were lost. In all 10 there was degeneration of renal tubular epithelium and it was questioned whether this was a true ante mortem change. For this reason 8 more rats were exposed on another occasion. Six showed necrosis of the inner cortical tubules of the kidneys. There was hepatic congestion in 4 and 4 showed congestion of the adrenal cortex. No lesions were seen in sections of brain, cord and sciatic nerve of 10 exposed and 4 control adult rats.

Part 2. Inhalation Exposures to 6.4 mg./liter (approximately 1500 p.p.m.). In table 1 is shown the result of a single inhalation exposure to 1500 p.p.m. of dichloroethane. There was a heavy mortality among guinea pigs, mice and rats of mixed sexes which were in the chamber for 7 hours. When the time of exposure was reduced to 4 hours and 2 hours, the mortality was decreased.

Repeated 7-hour inhalation exposures on consecutive days were then given to a group of animals (table 2). All of 29 rats died by the end of the fifth exposure. Twenty-one of these were females. Their fur became roughened after the first exposure and they developed an uncertain gait. There was failure of appetite and bloody crusts appeared about the nose. Some of the rats developed transitory coarse tremors. After 3 exposures the animals lay on their sides most of the time and moved with difficulty. Their respirations while in the chamber were extremely shallow.

At autopsy gross hemorrhage into the adrenal gland was a frequent finding. The lungs showed congestion and, in a few cases, hemorrhage. Microscopic study of 5 rats showed visceral congestion. In 4 others there was focal extravasation of blood in the lungs. Each rat showed degeneration and necrosis of renal tubular epithelium. The liver of one animal showed slight to moderate fatty degeneration. Unfortunately, sections of adrenal gland of all except 2 rats were lost. Both of these showed marked congestion with hemorrhage.

On another occasion microscopic sections were prepared from tissues of 12 rats which were similarly exposed. This time pulmonary congestion was noted for only 2 animals. Eight showed fatty degeneration of the myocardium of significant degree when compared with tissues of 6 control rats. Only 2 showed adrenal congestion. Degeneration or necrosis of the inner cortical renal tubules was noted for 7 rats. Three rats showed hepatic necrosis.

Nine young male guinea pigs all died after a few exposures. The animals showed lacrimation and inactivity while in the exposure chamber. At autopsy

TABLE 2
Mortality after repeated exposures to 1500 p.p.m. of dichloroethane

ANIMALS	NUMBER	AVERAGE WEIGHT	MOR- TALITY	TOTAL NUMBER OF DEATHS					
				Number of exposure days					
				1	2	3	4	5	6
		grams							
Rats	29	125	29/29	0	5	17	26	29	
Rabbits	5	1,640	4/5	1	1	1	2	4	
Guinea pigs	9	250	9/9	1	6	8	9		
Dogs	3		2/3	0	0	0	0	1	2
Hogs	2	32,300	2/2	1	2				

there was generalized visceral congestion which particularly involved the lungs. Microscopically, each of 7 pigs showed fatty infiltration of the myocardium. Four showed pulmonary congestion. Congestion of the adrenal cortex was noted in 2 pigs. All showed fatty infiltration of the renal tubules.

Five young rabbits, 4 females and 1 male, received repeated exposures to 1500 p.p.m. of dichloroethane. They appeared to be in good condition as far as one could observe, yet one after the other died—always during the night. Four were dead after 5 exposures. One rabbit survived 30 exposures.

Three male dogs were subjected to repeated exposures, each lasting 7 hours. After the second exposure two of the dogs refused to eat and became listless. After 4 exposures the same 2 dogs were quite ill. There was occasional emesis and the passage of red, watery stools which later became tarry. There were terminal transient convulsions and the dogs died in coma. One animal succumbed after 5 exposures and the other after 6 exposures.

Autopsy examination of the first dog showed congested lungs and a congested liver. The esophagus was markedly reddened at its cardiac end. The stomach

was full of changed blood, its mucosa was swollen and angry appearing and there were minute points of hemorrhage. The small bowel was congested while the large bowel was actually hemorrhagic. Microscopically there were auricular thrombosis with degeneration of auricular myocardium, pulmonary thrombosis with hemorrhage and edema of lung, moderate centrilobular fatty degeneration of the liver and focal degeneration of renal tubular epithelium.

The stomach of the second dog showed thickened rugae and marked congestion, but there were no bleeding points and it did not contain blood. Colon and lungs were also congested. Microscopically there was pulmonary congestion and hemorrhage, and very slight centrilobular fatty degeneration of the liver.

The third dog was able to survive 30 exposures without incident. Its appetite remained good and it appeared well. Gross and microscopic examination after sacrificing the animal showed nothing except the characteristic corneal opacity.

Histological sections of the brains of the 3 dogs showed only slight congestion.

Part 3. Results of exposure to lower concentrations. This will be the subject of another paper, for the work is not yet completed. The effect of repeated exposures to dichloroethane on other functions such as the hematopoietic system, or renal function and on hepatic function will also be reported at a later date. It may be stated here that repeated 7-hour exposures to 1000 p.p.m. were rapidly fatal to guinea pigs, rats and mice. Rabbits, cats, monkeys and dogs were able to survive much longer but eventually most of them succumbed. At 400 p.p.m. dogs and puppies remained in good condition for 8 months. Rabbits survived for nearly 100 exposures but eventually died. Guinea pigs and rats succumbed quickly. At 200 p.p.m. monkeys and rabbits appeared unaffected after 126 exposures, but deaths still occurred among guinea pigs and rats. Inhalation exposure studies using a concentration of 100 p.p.m. are in progress.

Part 4. Subcutaneous, oral and intraperitoneal injections. Table 3 gives data on the acute toxicity of dichloroethane for mice when administered orally, subcutaneously and intraperitoneally. We did not feel that it would be useful to gather more data of this kind on a larger series of animals because the results apply to only one particular strain of mice. The susceptibility of different strains obtained from the National Cancer Institute varied widely.

Table 3 also shows the acute toxicity of dichloroethane for young, adult Wistar rats receiving a single dose of the material subcutaneously. In another experiment repeated daily subcutaneous injections of 0.38 gms./kg. of dichloroethane dissolved in an equal volume of olive oil were given to 10 rats. The animals weighed between 70 and 90 gms. The rats lost weight, appeared listless and unkempt, and showed crusts about the nose. Nine of them died after the following number of injections respectively: 5, 6, 6, 13, 15, 15, 15, 15, 17.

Part 5. Effect of protective agents. Table 4 shows the effect of various chemical agents in protecting mice against death from inhalation of dichloroethane. The material being tested for protective action was given by stomach tube between 30 and 60 minutes before beginning the inhalation exposure to 400 p.p.m. of dichloroethane. For each test a separate group of control mice were used.

because in different experiments the strain of mice was not always the same. The "unprotected" mice received water, gum tragacanth solution or saline in order properly to match the various experimental groups. Most of the deaths occurred between 3 and 18 hours after the mice left the exposure chamber.

It will be noted that p-aminobenzoic acid afforded effective protection when given orally at a level of 2000 mg./kg. A dose of 1000 mg./kg. gave a smaller and quite variable degree of protection. Anthranilic acid (o-amino benzoic acid) was ineffective at 1000 mg./kg. and higher doses could not be used because of its own toxicity. The same held true for benzoic acid and sulfanilic acid. Sulfanilamide at a level of 1000 mg./kg. and aniline at a level of 500 mg./kg. were

TABLE 3
Acute toxicity of dichloroethane for mice and rats

SPECIES	AVERAGE WEIGHT	DOSE	SOLVENT	STRENGTH OF SOLUTION	ROUTE	MORTALITY IN 10 DAYS	MORTALITY
	grams	gm./kg		per cent			per cent
Mice	20.0	0.5	Olive oil	10.0	oral	0/6	0
Mice	19.0	0.6	Olive oil	5.0	oral	2/10	20
Mice	19.2	0.7	Olive oil	5.0	oral	6/10	60
Mice	20.0	0.8	Olive oil	5.0	oral	6/10	60
Mice	20.3	0.9	Olive oil	5.0	oral	10/10	100
Mice	20.0	0.25	Olive oil	50.0	s.c.	0/10	0
Mice	21.0	0.38	Olive oil	50.0	s.c.	8/10	80
Mice.	21.0	0.75	Olive oil	50.0	s.c.	10/10	100
Mice	24.9	0.25	Saline	0.6	i.p.	1/15	7
Mice	21.6	0.38	Saline	0.6	i.p.	5/16	31
Mice	21.1	0.44	Saline	0.6	i.p.	7/10	70
Mice	20.7	0.50	Saline	0.6	i.p.	12/15	80
Mice	20.0	0.62	Saline	0.6	i.p.	25/25	100
Rats	119.0	0.38	Olive oil	50.0	s.c.	0/4	0
Rats	89.0	0.50	Olive oil	50.0	s.c.	3/10	30
Rats	88.0	0.75	Olive oil	50.0	s.c.	5/10	50
Rats	95.4	1.00	Olive oil	50.0	s.c.	7/10	70
Rats	87.5	1.25	Olive oil	50.0	s.c.	8/10	80

protective. A group of amino acids were also tried. Of these, only methionine gave consistently good protection. Experiment No. 5 indicates that methionine was more effective than p-amino benzoic acid.

Methionine was also found to protect mice against the effects of intraperitoneal injection of dichloroethane. The mortality among 44 mice was 100 per cent when 0.5 cc. per kilogram of dichloroethane was injected. Seven out of 10 mice given an oral dose of 1500 mg./kg. of methionine survived the subsequent injection of dichloroethane.

Discussion. The data recorded here are in agreement with the somewhat scanty literature. Dichloroethane has been shown to be one of the more toxic of the halogenated hydrocarbons. With high concentrations of the vapor in air

TABLE 4

The influence of chemical agents on the inhalation toxicity of dichloroethane for mice

EXPERIMENT NUMBER	MATERIAL USED	DOSE	VOLUME IN- JECTED	NUMBER OF MICE	TOTAL NUMBER OF DEATHS					MOR- TALITY
					Days after exposure					
					0	1	2	7	10	
		mg./kg.	cc./kg.							per cent
1	Methionine	1500	50	8	0	0	0	0	0	0
	Methionine	750	25	8	0	2	2	2	2	25
	Water		50	8	3	6	6	6	6	75
2	Methionine	1500	50	10	0	0	0	0	0	0
	Cystine-HCl	1500	50	10	0	1	3	3	3	30
	Water		50	10	1	5	7	7	7	70
3	Methionine	1500	50	10	0	0	0	0	0	0
	Homocystine	2000	50	10	2	3	3	3	3	30
	1% Gum Tragacanth		50	10	4	9	9	9	9	90
4	Methionine	1500	50	9	1	1	1	1	1	11
	Water		50	9	6	7	9	9	9	100
	S-Methylisothiurea- sulfate	500	30	9	9	9	9	9	9	100
	Aniline	500	25	8	1	1	1	1	1	12
5	p-Amino benzoic acid	2000	25	8	0		6	6	6	75
	p-Amino benzoic acid	690	8.6	9	1		8	8	8	91
	Methionine	1500	50	8	0		0	0	0	0
	Methionine	750	25	8	0		6	6	6	75
	Ascorbic acid	2000	25	8	5		8	8	8	100
	Sodium Chloride	850	25	8	6		8	8	8	100
6	Cystine-HCl	1500	50	8	1	2	3	3	3	37
	Cystine-HCl	750	25	8	4	6	6	6	6	75
	Glycine	1500	50	8	4	5	5	6	6	75
	Water		50	8	5	6	6	6	6	75
7	p-Amino benzoic acid	500	5	9	1	5	6	6	6	66
	Methionine	500	20	9	0	2	3	3	3	33
	Sodium Chloride	500	50	9	2	7	8	8	8	89
8	p-Amino benzoic acid	2000	20	20	0	4	4	4	4	20
	Nothing			20	14	18	18	18	18	90
9	p-Amino benzoic acid	2000	20	15	1	4	4	4	4	27
	Nothing			15	5	11	11	11	11	73
10	p-Amino benzoic acid	2000	50	5	0	0	0	0	0	0
	Anthranilic acid	1000	25	8	1	7	7	8	8	100
	Sulfanilic acid	1000	25	8	3	8	8	8	8	100
	p-Amino benzoic acid	1000	25	8	0	4	4	4	4	50
	Sodium Chloride	850	25	8	3	6	6	6	6	75

TABLE 4—Continued

TABLE I. SUMMARY										
EXPERIMENT NUMBER	MATERIAL USED	DOSE	VOLUME INJECTED	NUMBER OF MICE	TOTAL NUMBER OF DEATHS					MORTALITY
					Days after exposure					
					0	1	2	7	10	
		mg/kg	cc/kg							per cent
11	p-Amino benzoic acid	2000	50	8	0	0	0	0	1	12
	Anthranilic acid	1000	25	8	2	7	7	7	7	88
	Sulfanilic acid	1000	25	8	5	8	8	8	8	100
	p-Amino benzoic acid	1000	25	8	0	0				
	Sodium Chloride	850	25	8	2	6	6	7	7	88
12	Aniline	500	25	8	1	1	1	1	1	12
	Anthranilic acid	1000	50	8	8	8	8	8	8	100
	Water		50	8	4	8	8	8	8	100
13	Sulfanilamide	1000	40	10	0	0	1	1	1	10
	Cystine	2000	40	10	0	7	8	8	8	80
	Homocystine	2000	40	10	0	3	4	4	4	40
	1% Gum Tragacanth		40	10	2	10	10	10	10	100
	Nothing			16	8	16	16	16	16	100
14	p-Amino benzoic acid	2000	20	15	0	0	0	0	0	0
	Methionine	1500	50	15	0	0	0	0	0	0
	Benzoic acid	2000	20	15	8	13	13	13	13	87
	Nothing			15	4	9	9	10	10	67

pulmonary congestion was a nearly constant finding. Kidney and liver injury was decidedly mild when compared with the effects of acute exposure to carbon tetrachloride or chloroform. Many of the animals developed adrenal cortical lesions.

It must be admitted that the exact mechanism of death in acute dichloroethane poisoning remains obscure. The situation is not comparable to delayed death in chloroform poisoning because no severe toxic hepatitis was noted. Some of the fatalities could be adequately accounted for on the basis of acute pulmonary edema. In other instances, however, the pulmonary lesions were minimal.

Various chemical agents were shown to protect mice against death from dichloroethane poisoning. These included methionine and various compounds containing an amino group in a benzene ring. The required dosage of protective agent was large. Many compounds, because of their own toxicity could not be administered in amounts comparable to those used for methionine and p-amino benzoic acid. For this reason no detailed study of the relation between chemical structure and effectiveness against dichloroethane poisoning was possible. Further work is under way which, it is hoped, will lead to the development of an agent useful in subacute dichloroethane poisoning.

It is of interest to point out that a protective action against other halogenated hydrocarbons has been attributed to some of these compounds. Leach and Forbes (11) found that sulfanilamide, sulfathiazole and sulfapyridine protected

rats against acute carbon tetrachloride poisoning, whereas sulfanilic acid was ineffective. Martin, Thompson and Accoustic (12) found that methionine was an effective detoxicant for tetrachlorethylene in mice; cystine and cysteine were less effective.

The protective action of methionine was emphasized in another paper of this series dealing with the influence of dietary factors on dichloroethane poisoning (13). Weanling rats were prepared for several weeks on a diet low in protein and choline and high in fat. Almost all of these rats, if untreated, would die after a 4-hour exposure to 1,000 p.p.m. of dichloroethane. Methionine or cystine, given orally just before the exposure, protected the lives of most of them. Choline was ineffective. Supplements of methionine added to the deficient diet were also protective.

Recently there was published a human case of carbon tetrachloride poisoning which, in the opinion of the authors, responded to treatment with methionine (14). Unfortunately there is no indication from our animal experiments that methionine could be used therapeutically in acute dichloroethane poisoning. Thirty-nine adult rats were exposed for 7 hours to 3,000 p.p.m. Two were dead at the end of the exposure period. Seventeen of the remainder received 1500 mg./kilogram of methionine intraperitoneally, 11 received water and 9 were given glycine. During the next 4 days all of the rats died. The animals were subjected to an exposure which caused death rather quickly. It is possible that in cases of subacute dichloroethane poisoning methionine would be of benefit, and this point is being investigated.

SUMMARY

(1) A 7-hour inhalation exposure to 12.4 mg./liter (3,000 parts per million) of 1,2-dichloroethane proved fatal to guinea pigs, rats, mice and rabbits. The animals showed varying degrees of narcosis while in the chamber. Death was preceded by dyspnea and increasing weakness. At autopsy there was pulmonary congestion, mild to moderate degeneration of renal tubular epithelium and occasionally necrosis of the adrenal cortex. Two raccoons and 3 cats were able to survive this exposure.

(2) Rats, mice, rabbits, guinea pigs, hogs and dogs were exposed to 6.4 mg./liter (1500 parts per million) of dichloroethane. Almost all of these animals succumbed before 6 exposures of 7 hours each were completed. Similar pathological findings were noted. No significant lesions were seen in brain or spinal cord of dogs or rats.

(3) The acute oral, subcutaneous and intraperitoneal toxicity of dichloroethane was determined for mice.

(4) Various chemical compounds given orally just before the inhalation exposure protected mice against the effects of dichloroethane poisoning. These included p-aminobenzoic acid, methionine, aniline and sulfanilamide.

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PHENOL CONJUGATION

IV. EFFECT OF SEVERAL INHIBITORS¹

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The effect of certain inhibitors on phenol conjugation has been studied partially by Bernheim and Bernheim (1), Lipschitz and Bueding (2), Hemingway, Pryde,

TABLE 1

Influence of monoiodoacetate on phenol conjugation by mammal livers in vitro

Medium: Krebs solution; pH = 7.2; phosphate buffer; phenol 5 mg. in 100 ml. Gas phase: air. Volume of solution in each flask 4 ml. Temperature 37.5°C.

ANIMAL	TOTAL PHENOLS	FREE PHENOLS	CONJUGATED PHENOLS	WET WEIGHT OF TISSUE	CONDITION
	γ	γ	γ	mg.	
Rat*	173	152	21	88	No MIA
	190	190	0	83	0.002 M MIA
	188	188	0	85	MIA + 0.001 M glucuronate
Guinea-pig	189	172	17	84	No MIA
	191	192	0	83	0.002 M MIA
	190	190	0	85	MIA + 0.001 M glucuronate
Dog (puppy)	182	165	17	90	No MIA
	180	182	0	84	0.002 M MIA
	188	187	1	92	MIA + 0.002 M glucuronate
Cat	196	178	18	83	No MIA
	190	191	0	91	0.002 M MIA
	200	200	0	86	MIA + 0.002 M glucuronate

* Rat tissues were incubated for 1 hour, the others for 90 minutes.

MIA = Monoiodoacetate.

and Williams (3), and ourselves (4, 5). With the purpose of gaining a better insight into the mechanism of conjugation several different compounds were tried, some of them effective as inhibitors on carbohydrate metabolism.

METHODS. The animals were killed by a blow on the head and the tissues were immediately removed and sliced according to the Warburg technique. The tissue slices were incubated at 37.5°C. in 50 ml. Erlenmeyer flasks, containing

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4 ml. Krebs (6) solution pH 7.2, phosphate buffer, with phenol added. Phenol concentration, incubation time and weight of tissue per flask are stated in the tables. Free and total phenols were determined according to the Theis and Benedict (7) method and calculated as micrograms in 4 ml. solution.

1. Influence of monoiodoacetate. The inhibition of phenol conjugation by monoiodoacetate has already been shown (5) in one strain of rat. It also in-

TABLE 2
Influence of several inhibitors on phenol conjugation by rat liver in vitro
Medium: Krebs solution; pH = 7.2; phosphate buffer. Gas phase: air. Volume of solution in each flask 4 ml. Incubation time 1 hour. Temperature 37.5°C.

INHIBITOR	TOTAL PHENOLS	FREE PHENOLS	CONJUGATED PHENOLS	WET WEIGHT OF TISSUE	CONCENTRATION OF INHIBITOR	PHENOL ADDED
Azide	7	7	7	%		7
	185	164	21	88	No azide	200
	194	194	0	83	0.02 M azide	200
	193	192	1	83	0.01 M azide	200
	186	186	0	88	0.005 M azide	200
Fluoride	190	184	6	85	0.005 M azide	200
	195	185	10	80	0.0025 M azide	200
	39	31	8	116	No NaF	200
	35	36	0	96	0.02 M NaF	40
	35	35	0	104	0.01 M NaF	40
Fluoride*	39.5	40.5	0	88	0.005 M NaF	40
	38	36.5	1.5	99	0.001 M NaF	40
	39	39	0	55	0.01 M NaF	40
	37	38	0	52	0.005 M NaF	40
	37	37	0	65	0.002 M NaF	40
Octyl alcohol†	38	29	9	40	Not added	40
	32.5	32.5	0	35	Added	40
	38	24	14	25	No NaHSO ₄	40
Sodium bisulfite	33	33	0	30	0.0005 M NaHSO ₄	40
	29	29	0	25	0.0004 M NaHSO ₄	40
	29	29	0	30	0.0003 M NaHSO ₄	40
	33	29	4	25	0.0002 M NaHSO ₄	40
	43	29	14	30	0.0001 M NaHSO ₄	40

* Krebs solution without calcium.

† Krebs solution saturated with octyl alcohol.

inhibits the formation of conjugated glucuronic acids as shown by Lipschitz and Bueding (2). The results obtained with livers of the Vanderbilt strain (8) and other species of mammals are shown in table 1. All the species studied behaved similarly, 0.002 M monoiodoacetate inhibits conjugation completely and there is no recovery upon the addition of 0.001 to 0.002 M glucuronate.* The previous feeding

* Neutralized glucurone was used.

of phenol to rats has no effect on the inhibition by this drug. Increasing the glucuronate concentration does not affect the results.

2. *Influence of other inhibitors.* It has already been shown that cyanide inhibits phenol conjugation (1, 2, 3, 4). As azide acts in a similar way it also should inhibit. Table 2 shows that azide completely inhibits phenol conjugation down to a concentration of 0.01 M.

Octyl alcohol, which is known to inhibit certain enzymes also completely inhibits the conjugation of phenol.

Fluoride and bisulfite are also inhibitors of phenol conjugation. A complete inhibition is produced by 0.005 M fluoride and by 0.0003 M bisulfite. The activity of fluoride is approximately the same whether calcium is present or not. A slight decrease in conjugation seems to occur in absence of calcium, though more experiments are necessary to decide this point. Lack of magnesium has no influence on conjugation.

0.02 M sodium oxalate partially inhibits phenol conjugation, whereas sodium citrate has no effect.

It has not been possible to study the effect of malonate as it interferes with phenol determination.

I am indebted to Associate Professor Frederick Bernheim for criticism and to Dr. E. Bueding for the glucurone used in the present work.

SUMMARY

1. 0.002 M moniodoacetate inhibits phenol conjugation in rat (Vanderbilt strain), guinea-pig, dog (puppy), and cat liver, which is not reestablished by addition of glucuronate.

2. Phenol conjugation is also inhibited by 0.01 M azide, octyl alcohol, 0.005 M fluoride, 0.0003 M bisulfite and partially by 0.02 M sodium oxalate.

3. Lack of magnesium and presence of citrate have no effect on conjugation.

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SYNTHETIC ANTICONVULSANTS

5,5-DISUBSTITUTED HYDANTOINS CONTAINING A HETERO-ATOM IN THE SIDE CHAIN

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The promising anticonvulsant effect of certain 5-alkoxymethylhydantoin observed in preliminary experiments in the cat (1) encouraged a concerted study of anticonvulsant properties of other hydantoin containing a hetero-atom in the side-chain. Through this cooperative, extensive, study of substituted hydantoin, it has been possible for us to determine (a) the anticonvulsant effect induced when the phenyl group is substituted for an alkyl group in the alkoxy-methylhydantoin (compare I, II and III, figure 1); (b) the effect of reducing the phenyl group to cyclohexyl (III and IV); (c) the replacement of oxygen by sulfur in the phenyl alkoxy-methylhydantoin (III and V); and (d) the activity after oxidation of the sulfur atom to the corresponding sulfone in the phenyl alkythio-methyl series (V and VI).

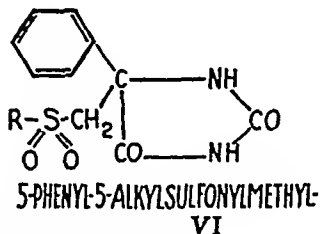
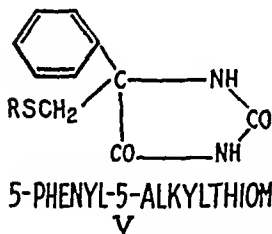
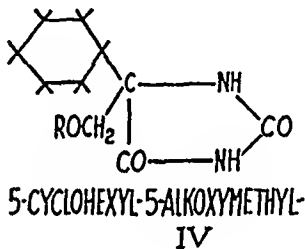
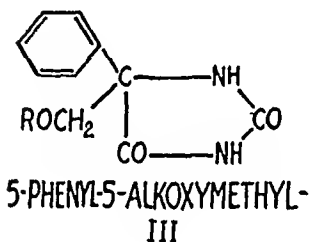
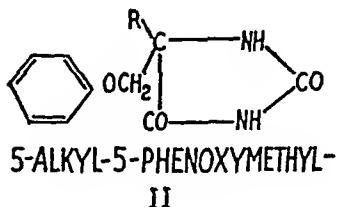
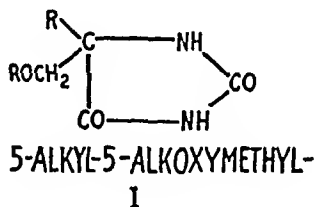
The 5-alkyl-5-alkoxymethyl-, (2) 5-alkyl-5-phenoxy-methyl-, (3) (4), 5-phenyl-5-alkoxymethyl-, (5), and 5-cyclohexyl-5-alkoxymethyl-hydantoin were prepared by Professor Henry R. Henze and his co-workers at the University of Texas. The 5-phenyl-5-alkythiomethyl-, and 5-phenyl-5-alkylsulfonylmethyl-hydantoin were synthesized by Dr. L. M. Long of Parke, Davis and Company (6). A comprehensive list of carbonyl compounds which have been converted to hydantoin by Professor Henze is available as Document No. 1603 of the American Documentation Institute. The melting point of many of the compounds used in this study are described in it.

EXPERIMENTAL. The anticonvulsant effect was measured in cats by the method previously described (7, 8). By this method, the strength of current necessary to produce a convulsion was determined immediately before and approximately two hours after oral administration of the compound. The convulsive threshold in animals (cats) weighing 2 to 4 kilos was usually in the range of 15 to 25 ma. After administration of an effective anticonvulsant such as dilantin sodium or phenobarbital, this threshold was raised to over 50 ma. In order to conserve animals, stimulations in excess of 50 ma. were not given. The anticonvulsant action of the compounds was rated as follows:

- 0—No change in convulsive threshold.
- +—Elevation of convulsive threshold by 5 to 15 ma.
- ++—Elevation of convulsive threshold by 20 to 30 ma.
- +++—Convulsive threshold elevated to 50 ma.
- ++++—Convulsive threshold elevated to more than 50 ma. (i.e., No convulsion obtained when stimulated with 50 ma.)

The hydantoins were usually dissolved in one equivalent of dilute sodium hydroxide solution and administered orally by stomach tube unless otherwise noted in the tables.

RESULTS. As shown in table I, the promising activity originally observed with 5-ethyl-5-isoamoxymethylhydantoin was not obtained upon subsequent testing. Moreover, the other two prototypes tested were also relatively in-



(R = METHYL, ETHYL, PROPYL, ETC)

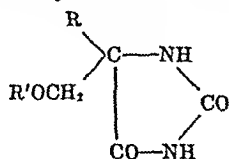
FIG 1. HYDANTOINS

active. Replacement of the alkyl group by a phenyl group in the R' position likewise did not induce activity although some effectiveness was obtained in relatively high doses. However, substitution of the R, or alkyl group, by a phenyl group and varying R', resulted in greatly increased activity (compare Table 1 and 2 (x = 0)). It can be readily seen that the 5-phenyl-5-alkoxymethyl series has consistently induced a rise in the convulsant threshold of the cat in low doses, with the exception of the hexyl derivative.

Substitution of the oxygen atom in the side-chain by sulfur did not change the qualitative effectiveness of the parent oxygen homologues although there is some quantitative difference. However, when one compares the results obtained with the methyl, ethyl, isopropyl, and n-amyl derivatives, it can be seen that the oxygen compounds are more potent. Further investigation of thiomethyl derivatives is in progress. In both series the n-hexyl compounds were inactive. There is no explanation at present for the fact that 5-phenyl-5-n-hexylsulfonylmethylhydantoin and its iso-butyl homologue raised the convulsive threshold in doses

TABLE 1

Anticonvulsant activity of 5-alkoxymethyl and 5-phenoxyethyl-5-alkylhydantoins



R	R'	ACTIVITY*
Ethyl	Iso-Amyl ^a	0, 50 ^b ; 0, 100; 0, 135; 0, 315
Iso-Amyl	Methyl	0, 40; 0, 90; ±, 140; + + + +, 250
Iso-Amyl	n-Propyl	0, 20; 0, 40; 0, 65 ^c ; 0, 80; 0, 240 ^e
Methyl	Phenyl	0, 140; + + +, 175 ^d
Ethyl	Phenyl	+ + +, 140 ^d
Iso-Propyl	Phenyl	0, 87; 0, 80; 120 ^f
n-Amyl	Phenyl	0, 70; 0, 250; +, 400
Iso-Amyl	Phenyl ^g	0, 90; 0, 125; + + + +, 280; + + +, 400 ^d
Phenyl	Phenyl	+ + + +, 120; + + + +, 100 ^e

* The anticonvulsive activity varying from 0 to + + + + is given for the dosage expressed in milligrams per kilogram of body weight of the animals.

^a Henze (2) reported preliminary results which showed this compound to be unsatisfactory as a soporific.

^b Reference (3).

^c Convulsant. The cat given 65 mg/kg. died in status a few minutes after treatment.

^d Administered orally in capsules.

^e Sodium salt given in aqueous solution.

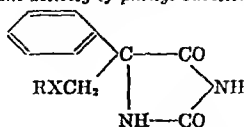
^f Fatal.

^g Minimum effective anticonvulsant dose.

of 130-140 mg./kg., whereas other homologous sulfones were not active at that level. Reduction of the phenyl group of the 5-phenyl-5-alkoxymethylhydantoins to cyclohexyl resulted in loss of activity. (Compare table 2, X = O, with table 3.)

CONCLUSION. The most promising compounds found in this study, at least from the standpoint of anticonvulsant activity with a minimum of hypnotic effect were 5-n-propoxymethyl- and 5-iso-propoxymethyl-5-phenylhydantoin. These were, therefore, subjected to more extensive study. The ability to afford sustained protection against electrical stimulation was established for each drug.

TABLE 2
Anticonvulsant activity of phenyl substituted hydantoins



R	x = O		x = S		x = SO ₂	
	MLD† mg./kg.	Activity*	MLD† mg./kg.	Activity*	MLD† mg./kg.	Activity*
Methyl	400 ^a	0, 37; + + +, 63; + + + +, 74		0, 75; + + +, 150		0, 80; 0, 130; + + + +, 200
Ethyl	450 ^a	0, 22; 0, 27; + + + +, 33; + + + +, 42	>500	0, 100; + + + +, 150; + + + +, 200		0, 25; 0, 60; 0, 115
n-Propyl	500	+ + +, 45; + + + +, 70	330 ^a	0, 55; + + + +, 100	900	0, 65; 0, 170
iso-Propyl	200	0, 15; + +, 20; + + + +, 27; + + + +, 35	450 ^a	0, 30; + +, 70; + + +, 110	900	0, 30; 0, 90; 0, 150
n-Butyl	250 ^a	+ + + +, 88; + + + +, 90	300±	+ , 45, + + + +, 60; + + + +, 110		0, 50; + +, 120
iso-Butyl	350 ^a	+ , 68; + + + +, 88		+ +, 70, + + + +, 140		+ , 75; + + + +, 130
s-Butyl	350	0, 17; + + + +, 45; + + + +, 75				
t-Butyl	400	+ , 45; + + + +, 100				
n-Amyl	300	+ , 21; + + + +, 75	250 ^a	+ + +, 75; + + + +, 150	500	0, 110; 0, 180
iso-Amyl	250 ^a	+ + +, 45; + + + +, 100				
s-Amyl	350 ^a	+ + +, 73, + + + +, 140				
1-Ethylpropyl	350 ^a	+ , 45; + + + +, 60				
1,2-Dimethylpropyl	100 ^a	+ , 40, + + + +, 75				
2-Methylbutyl	350	+ +, 80, + + + +, 116				
n-Hexyl	300 ^a	0, 40; 0, 120 ^b	250 ^a	0, 50, 0, 110; 0, 175	600	+ +, 70; + + +, 140
Cyclohexyl		0, 30, 0, 70; 0, 110	250	+ + +, 33, + + + +, 75	1000	0, 50; 0, 100
Phenyl	1750 ^c	0, 35; + + + +, 100; + + + +, 120		0, 20, 0, 50; 0, 250		0, 25; 0, 40, 0, 180
Benzyl.			330	+ + + +, 120	600	0, 70; 0, 200

* The anticonvulsive activity varying from 0 to + + + + is given for the dosage expressed in milligrams per kilogram of body weight of the animals

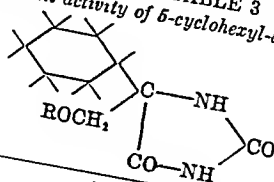
† Intraperitoneal injection in white mice. We are indebted to Mr. L.W. Rowe for this data. The M.L.D. recorded here is an approximate LD₅₀

^a Hypnotic in lower doses.

^b Administered in propylene glycol.

^c Orally to white mice.

TABLE 3
Anticonvulsant activity of 5-cyclohexyl-5-alkoxymethylhydantoins

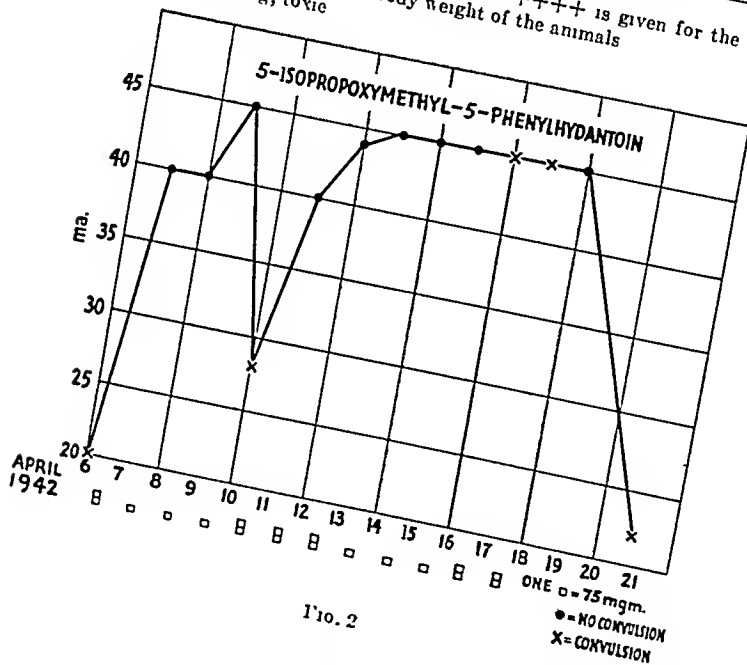


R	MLD*	ACTIVITY†
Methyl	600	0, 75; +, 110, +, 133
Ethyl	300	0, 30; 0, 75‡; 0, 100
n-Propyl	350	0, 45; +, 60
iso-Propyl	400	+, 70, ++, 75
n-Butyl	340	0, 40; 0, 75
iso-Butyl	150	+, 35; +, 85
sec-Butyl	400	0, 30; 0, 60
n-Amyl	350	+, 110; +, 200
iso-Amyl	450	0, 110; +, 160
s Amyl	550	0, 80; 0, 180; 0, 188
1 Ethylpropyl	600	0, 100, 0, 160
1,2-Dimethylpropyl	100	0, 110, +, 160
n-Hexyl	250	0, 38; 0, 60
Cyclohexyl		0, 50, 0, 110

* By intraperitoneal injection to white mice

† The anticonvulsive activity varying from 0 to ++++ is given for the dosage expressed in milligrams per kilogram of body weight of the animals

‡ Cat limp and drooling, toxic



First the basal threshold of each animal was established and in addition the threshold was determined each morning before the daily administration of the drug throughout the experiment. The results (figures 2 and 3) show that with a dose varying between 75-150 mgms. daily, the threshold was consistently elevated throughout the period of testing. The x's on the chart indicate the minimum level necessary for the production of a convulsion; the o's, that no convulsion was obtained with the amount of current designated. The iso-propyl derivative afforded a more consistent and lasting rise in the convulsive

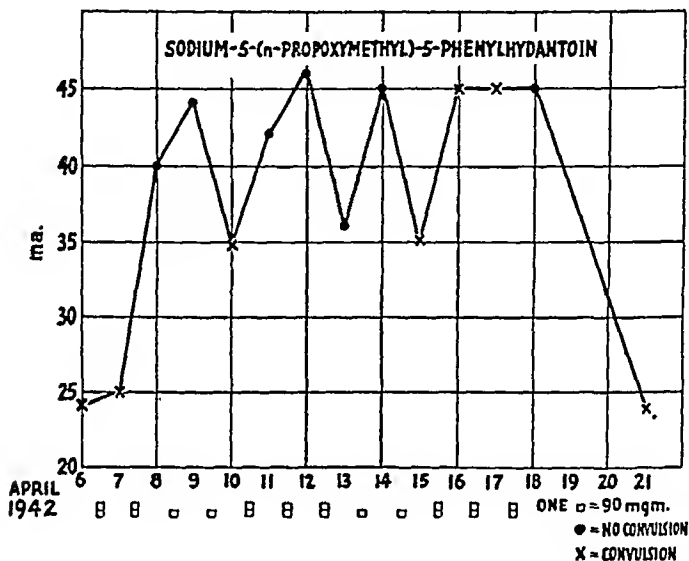


FIG. 3

threshold. The acute toxicity of this drug for cats was determined by Mr. L. W. Rowe who, after feeding it to 25 cats, reported the LD_{50} orally to be 462.5 mg./kg. The therapeutic ration $\frac{(LD_{50})}{(MED)}$ therefore, is 13. On the basis of the above information, the iso-propyl compound is recommended for clinical trial.

SUMMARY

A series of sixty-two 5,5-disubstituted hydantoin containing oxygen and sulfur in one alkyl side-chain, have been tested for anticonvulsant activity in the cat. The 5-phenyl-5-alkoxymethyl- and 5-phenyl-5-alkylthiomethylhydantoin possess pronounced anticonvulsant activity. Two derivatives 5-phenyl-5-n-propoxymethyl- and 5-phenyl-5-iso-propoxymethylhydantoin were studied chronically in cats. The latter derivative affords full protection against electrically induced convulsions in doses of 33-37 mg./kg. in acute experiments

and gives a lasting and consistent rise in the convulsive threshold in chronic tests. This compound is therefore recommended for clinical trial.

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SUSCEPTIBILITY OF BIRDS TO INSULIN AS COMPARED WITH MAMMALS

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It is well known that ducks and chickens withstand surgical removal of pancreas better than mammals (1-7). Their induced diabetes by such a procedure is usually mild and temporary. It is also a fact that pigeons, fowls, ducks, hawks, and quails have a high resistance to insulin, requiring larger doses to produce convulsions than mammals (8-15). The present investigation was part of our study on the species difference of response to drugs. It consisted of a comparison of the convulsive action of insulin, under almost identical conditions, in canaries, pigeons, Pekin ducks, white Leghorn roosters, mice, rats, rabbits, and dogs.

Healthy animals, housed in the same building, the temperature of which fluctuated between 26.7 and 28.9°C., were starved for approximately 16 hours before insulin was administered. In order to avoid any difference in absorption, the hormone was injected intravenously: by the tail vein in mice and rats; by the wing vein in canaries and pigeons; by the marginal vein of the ear in rabbits; and by the saphenous vein in the remaining animals. Graded doses were employed for 4-6 groups of each species of animals, so that the median convulsive doses (CD_{50} 's) could be satisfactorily determined. Each group varied from a minimum of 3, to a maximum of 17, animals. All dilutions of insulin were made from the same stock solution, 1 cc. of which equaled 500 units. Convulsions were watched for in the injected animals during the working day, 8 a. m. to 5 p. m., but not throughout the night. A number of animals were found dead the next morning, and were presumed to have convulsed if convulsions had already been established for that species. Blood sugars, according to the method of Hagedorn and Jensen (16), were determined on 5 ducks and 10 roosters at frequent intervals. Once convulsions occurred, the animal was discarded.

The results obtained with 7 species of animals are summarized in table 1. It is obvious that the birds, as a whole, are less susceptible to insulin than the mammals. Among the birds tested, there is a wide difference in sensitivity—the duck being the most sensitive, and the rooster, the least sensitive. Some ducks convulsed in 2 hours after receiving adequate doses of insulin, but the majority of them, much later. Blood sugar curves indicate that their shock level is not far from that of mammals, 41-50 mg. per 100 cc. or less. The pigeon is about 4 times as tolerant to insulin as the duck, as judged by the CD_{50} 's. In most cases, it took more than 24 hours for convulsions to take place. The canary, in turn, is about 3 times as tolerant as the pigeon. In this species, a few animals receiving effective doses of insulin showed definite convulsions in 30 minutes, but most of them died overnight, presumably following convulsions.

The rooster was exceptional, in that none in our series developed convulsions during the periods of observation. The results (not listed in the table) may be given as follows: Of 9 roosters injected with varying doses from 100-500 units per kg., 2 fell on side, 1 had ataxia, but all appeared normal the next day; and of 9 others injected with 1000, 2000, and 5000 units per kg., 3 each, 6 died in 28-72 hours. For illustration, the blood sugar curves of 4 roosters are shown in figure 1. Only 1 animal receiving 2000 units per kg. had marked and protracted hypoglycemia, but ultimately recovered. It appears that death from insulin in roosters may not be necessarily due to hypoglycemic shock. Our failure in observing convulsions in this animal is confirmatory of the published work of Cassidy, Dworkin, and Finney (11). Golden and Long (15), on the other hand, succeeded in producing convulsions and eventual death of chicks by administration of insulin on successive days.

TABLE 1
Convulsive dose of insulin in birds and mammals

ANIMAL	BODY WEIGHT RANGE	NUMBER USED	DOSE RANGE	CD ₅₀ ± STANDARD ERROR
	kg		units per kg	units per kg.
Canary	0.0163 ± 0.0002	24	1000-4000	2396 ± 321
Pigeon	0.2998 ± 0.0036	67	400-1200	705 ± 102
Duck	2.1890 ± 0.038	26	50- 500	157 ± 41
Dog	7.030 ± 0.230	35	16- 30	23.3 ± 3.4
Rat	0.0985 ± 0.0016	60	9- 36	20.7 ± 0.2
Mouse	0.0156 ± 0.0002	60	7- 40	17 ± 3.0
Rabbit.	1.891 ± 0.033	49	4- 8	5.1 ± 0.45

Among the mammals, the rabbit is fully 3-4 times as susceptible as the mouse, rat, and dog. The speed of reaction is relatively more rapid in the mouse and rabbit, for convulsions in them appeared in 45-120 minutes after administration of effective doses, while those in the rat and dog took place in 2-6 hours. In no instance was the response immediate, even though the hormone was given intravenously. The presence of a latent period is characteristic of insulin action.

Compared with the birds, the rabbit may be said to be about 30 times as sensitive to insulin as the duck, 138 times as sensitive as the pigeon; and 470 times as sensitive as the canary. As a whole, the speed of reaction is slower in birds than in mammals. Allen (17) gave figures to indicate that man was more sensitive to insulin than the rabbit, mouse, and rat, but less sensitive than the dog. Since the present results show that the rabbit is more sensitive to insulin than the dog, it is desirable to determine more precisely the position of man regarding his susceptibility as compared with lower mammals by a single intravenous injection.

How one can account for the relatively high resistance of birds to insulin—over-supply or deficiency—will depend upon further elucidation of their carbohydrate metabolism. Published data show that the latter deviates from that

of the mammals. Both the chicken and the duck have a lower unitage of insulin in their pancreas than the rabbit, dog, mouse, and rat (18), indicating, perhaps, that they are able to carry on their body oxidation with less insulin. Phlorizin produces much more accumulation of acetone bodies in the blood of the de-pancreatized dog than that of the normal dog. With the duck, however, the reverse is true (7). The rate of glycogenolysis in the excised liver tissue of the pigeon is certainly slower than that of the mammals (19). The young, starved rabbit deposits glycogen in the liver following the administration of insulin

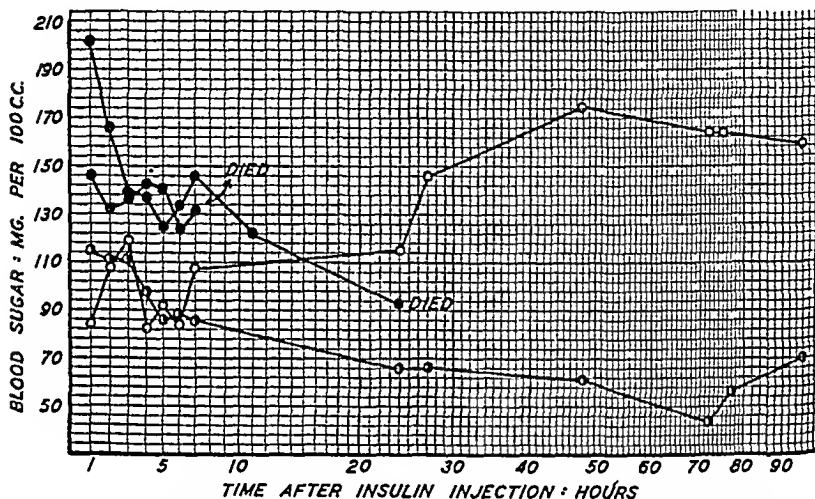


FIG. 1. BLOOD SUGAR OF ROOSTERS AFTER INSULIN INJECTION

Curve through hollow circles was the animal receiving 200 units per kg.; that through semi-solid circles, 2000 units per kg.; and those through solid circles, 5000 units per kg

(20), while the fasting chicken does not show the same response (21). The absence of typical convulsions after excessive doses of insulin also suggests that the chicken's central nervous system is especially tolerant to this stimulating phase of insulin action.

SUMMARY

By intravenous injection of insulin, the canary, pigeon, duck, and rooster are shown to be more resistant than the mouse, rat, rabbit, and dog, as judged by the convulsive dose, speed of reaction, and blood sugar determinations in the duck and rooster. Among the birds, the duck is the most, and the rooster, the least sensitive. The latter fails to develop convulsions even with lethal doses of insulin.

Acknowledgment. The authors are indebted to Messrs. John C. Hanson, Harold M. Worth, Clarence E. Powell, and Miss Marian H. Ellaby for their invaluable assistance in these experiments.

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THE CHEMICAL BASIS OF MARIHUANA ACTIVITY¹

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Although great progress has been made in isolating the marihuana-active tetrahydrocannabinols from hemp (10), in synthesizing congeneric dibenzopyran derivatives and in determining the structure-activity relationship in this class of chemicals (1, 5), the question of the interrelation between the two main pharmacological actions of hemp, motor ataxia and corneal areflexia, still remains obscure.

The original assumption (4) was that the corneal areflexia action in the rabbit has the same chemical basis as the ataxia action in the dog, and some students of the subject (8) still consider it as reliable an indicator of the psychic action in man as the ataxia action. These assumptions have yet to be put to the test. Some studies have been made in an attempt to determine whether or not the ratio of areflexia to ataxia potency is the same in different hemp preparations (3, 5) and whether it remains unchanged when they are subjected to chemical alterations (3). Such studies can contribute to a solution when based upon accurate potency measurements. This technical prerequisite is fulfilled for the assay of ataxia in the dog (6, 9, 5), but there is as yet no equally reliable method for the assay of areflexia, the response of the rabbit, the only test animal available, being extremely inconstant (7, 5).

The present study attempts to overcome this obstacle and to elucidate the relationship between the two marihuana actions by comparing the areflexia and ataxia potencies of an impure hemp preparation and of a pure ataxia-active substance before and after subjecting them to mild oxidation.

EXPERIMENTAL. A crude ethanol extract from Oriental hemp resin (charas) and the highly ataxia-potent tetrahydrocannabinol prepared from the same resin (10) were employed in these experiments.² Parahexyl, a synthetic racemic *n*-hexyl homolog of tetrahydrocannabinol (2), was employed as standard in all areflexia and ataxia assays. This substance had a potency twice that of the synthetic racemic tetrahydrocannabinol employed as the standard in previous studies (5).

For oxidation, aliquot parts of the standard ethanol solution of each of these preparations were aerated in a current of washed air for six hours at 60° C. Solvent lost by evaporation was replaced at the end of the aeration. All four test preparations were injected intravenously in dilute solutions of ethanol.

Ataxia assays were conducted by the "method of approximation" (6, 9, 5) in dogs which before and after each assay were repeatedly tested with the standard. Corneal areflexia activity was assayed in rabbits. Intensity, measured by the incidence of lid responses to ten touches of the central cornea with a calibrated hair, and duration of the anesthetic

¹ This work was supported in part by a grant from Abbott Laboratories, Inc., North Chicago.

² Both preparations were obtained from the Narcotics Laboratory, U. S. Treasury Department, Washington, D. C., through the courtesy of Dr. H. J. Wollner.

effect were taken into account. The great individual differences in sensitivity were controlled in the same way as in the ataxia assays, i.e., by alternately testing the unknown and the standard preparation in the same animal as described in a previous paper (9). In order to eliminate error due to change in sensitivity with time the data were used only when it was established by repeated tests with the standard preparation in the same animal that they were from a period of constant sensitivity.

In a group of 15 rabbits tested about 500 times during a period of six months about one-half the animals had one or more periods of constancy of from two to eleven weeks duration in which data were obtained suitable for the estimation of areflexia potency. 48 tests of the unknowns and 39 of the standard carried out during these periods served to determine the areflexia potency of the four preparations. Their ataxia potency was determined by means of 87 tests in dogs.

TABLE 1

Influence of oxidation upon ataxia and areflexia potency of a pure tetrahydrocannabinol and of a mixture of hemp components

	AREFLEXIA			ATAXIA			PO- TENCY RATIO*	PER CENT DE- CREASE IN PO- TENCY BY AERATION	
	Number of tests	Potency		Number of tests	Potency			Are- flexia	Ataxia
		Average	Range		Average	Range			
Charas extract Aerated	21	1.10	>1.00-<1.20	6	0.45	>0.40-<0.50	2.44	70	0
	12	0.33	>0.30-<0.35	9	0.50	>0.40-<0.60	0.67		
Charas tetrahy- drocannabinol Aerated	8	1.23	>1.00-<1.45	67	5.54	>4.84-<6.16	0.22	0	0
	7	1.22	>0.97-<1.48	5	5.43	>3.96-<6.90	0.23		

* Areflexia units per unit of ataxia potency.

As is seen from the assay results before and after aeration (table 1), pure charas tetrahydrocannabinol is both ataxia and areflexia active, and is not affected by mild oxidation; both actions are retained undiminished. The experiments also establish the standard preparation, parahexyl, as another example of a pure substance capable of both actions. The crude charas extract shows about the same areflexia potency as the pure tetrahydrocannabinol, but only one-eleventh the ataxia activity. Whereas oxidation did not change the ataxia potency of the crude extract, it reduced its areflexia potency to one-third.

COMMENT. There is now ample proof of the fact that the two experimental manifestations of systemic marihuana-activity, motor ataxia and corneal areflexia may reside in the same molecule. The present study adds two such substances, natural charas tetrahydrocannabinol and synthetic parahexyl, to two formerly reported examples, namely, two semi-synthetic tetrahydrocannabinols (5). Many more synthetic congeners in this class of benzopyrans were, when first described (1, 5), reported to be ataxia-active, and later (8) shown to possess also areflexia activity. As all strains of hemp contain mixtures of different tetra-

hydrocannabinols, and as in all the above pure tetrahydrocannabinols the ratio between the two activities is different, the inconstancy of the areflexia ataxia potency index in impure hemp preparations is entirely compatible with the concomitance of the two activities in one molecule.

However, the present assays show that representation in the same substance is not the complete solution of the problem and that hemp, in addition, contains areflexia principles of a different nature. The varying ataxia and, disproportionately with it, areflexia activity of impure preparations may or may not be due solely to the presence of different mixtures of various tetrahydrocannabinols. Either a molecule having exclusively areflexia action or only the prosthetic group responsible for areflexia in a molecule having also a group responsible for ataxia might be altered by an oxidative process which reduces areflexia, but not ataxia activity. No clue to these alternative possibilities is provided by the earlier experiments (3, 5). The present experiments give evidence that a mild oxidation leaves the areflexia potency of charas tetrahydrocannabinol unchanged, but reduces by 70 per cent that of the crude preparation from which it is obtained. In both preparations, as in the extracts formerly (3) subjected to a similar procedure, the ataxia activity remained unchanged. This is proof that other ataxia active natural tetrahydrocannabinols are as resistant to oxidation as this charas tetrahydrocannabinol. Therefore the susceptibility of hemp extracts to oxidation must reside in areflexia active substances having little or no ataxia activity.

Thus hemp presents the unusual phenomenon that two important biological actions of a drug are in part combined in the same molecule and in part embodied independently in separate molecules. While the identification of the substances of the former type, the areflexia and ataxia active isomeric tetrahydrocannabinols, is well advanced, the principles having areflexia activity alone are as yet unidentified. They may be either unknown tetrahydrocannabinols or compounds of entirely different structure.

Since the two actions are shown to be due partly to different substances, mixtures of marihuana active substances cannot be evaluated for their content in ataxia principles by areflexia assays and vice versa. Moreover, since tetrahydrocannabinols vary in the relative intensity of the two activities, they cannot form the same sequence when grouped in the order of their areflexia potency as when grouped according to ataxia potency. While much is known regarding the relationship between structure and ataxia activity (1, 5), the relationship between structure and areflexia activity has not yet been investigated.

The demonstration of a dissociation between the two manifestations of marihuana activity raises a new problem with respect to the third action which is manifest in the psychic effects in man. Some data suggest a certain parallelism between this action and ataxia activity, but nothing is known about the relationship between psychic action and areflexia. This study indicates that psychic action in man, if paralleled by ataxia in dogs, cannot be evaluated in terms of areflexia action. But it also shows the way to the solution of the problem. It offers two preparations with different potency indices, one with

an areflexia potency eleven times higher in relation to ataxia potency than the other. Evaluation of the relative effectiveness of these two preparations in man should answer the question of the interrelation between the three chief pharmacological actions of marihuana.

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EFFECTIVENESS OF CAFFEINE (1,3,7 TRIMETHYLYXANTHINE) AGAINST FATIGUE

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Foltz, Ivy, and Barborka (1942, 1943) demonstrated that in the human subject caffeine increases not only the capacity for muscular work in rested individuals, but also the speed of muscular recuperation in fatigued subjects. It is obvious that in human experiments it is impossible to determine whether the caffeine achieves its effect through a central mechanism, acts on the neuromuscular junction, or stimulates the muscle directly. The classical textbooks of pharmacology fail to clarify these considerations. The experiments which have been done, in the past mostly on frogs, indicate that the xanthine derivatives have a direct action on muscle itself (Goodman and Gilman, 1941) and that they are even capable of producing an increase of muscular contraction when a muscle is stimulated indirectly through its nerve (Zunz, 1932). It seemed of interest to us to learn whether caffeine has a de-fatiguing action in mammals and to determine, in case there is such an action, what is its mechanism. The object of the present study is to clarify these points.

METHODS. The animals used were cats anesthetized with Dial (0.75 cc. of Dial Ciba per kilo body weight, intraperitoneally). In some experiments the animals were decerebrated or an Elliott preparation (pithed preparation) was made during a short etherization. A tracheal cannula was inserted in the event that artificial respiration should become necessary.

The studies were conducted principally on the quadriceps femoris muscle, though the soleus was also used; both were stimulated indirectly through the nerve, previously isolated and sectioned, by means of condensers controlled through vacuum tubes. Maximum stimuli were applied. Silver electrodes insulated with rubber were used. The muscular excursion was recorded by fixing the femur (in the case of the quadriceps femoris) or the tibia (in the case of the soleus) with clamps, and attaching the tendon to the short end of a lever which then stretched elastic bands.

In some instances the muscles were stimulated directly. For this a preliminary denervation was done under ether anesthesia 3 to 7 days prior to the experiment, and needles were used as electrodes. One needle was applied to the tendon and the other in the muscle belly. Maximum stimuli were always used. In some other cases the muscles were stimulated with acetylcholine. This was injected either into the femoral artery or into the abdominal aorta (after a preliminary ligation of the contralateral iliac artery and the median sacral artery). The amount used was 10 to 40 mg. of acetylcholine in a volume not greater than 0.30 cc., usually about 0.20 cc. In order that the muscles might be more sensitive to acetylcholine action they were denervated 5 to 15 days prior to the experiment.

The nictitating membrane was also used. Recordings were made after a preliminary enucleation of the eye and ligation of the external carotid artery. This organ was stimulated by means of an intracarotid injection of 40 to 60 mg. of acetylcholine in a volume not greater than 0.30 cc., generally 0.20 cc.

In some animals the arterial blood pressure was measured. For this purpose either a mercury manometer or a manometer of membrane was utilized, either being connected with the common carotid artery.

Drugs used in the experiments were: acetylcholine hydrochloride (Roche), prostigmine (Roche), caffeine (1, 3, 7 trimethylxanthine, 3.5 gm. dissolved in 100 cc. of a 5% solution of sodium benzoate), 5% sodium benzoate, Brazilian curare (crude product), and atropine sulfate (Merck). Some of these drugs were injected into the femoral artery above the

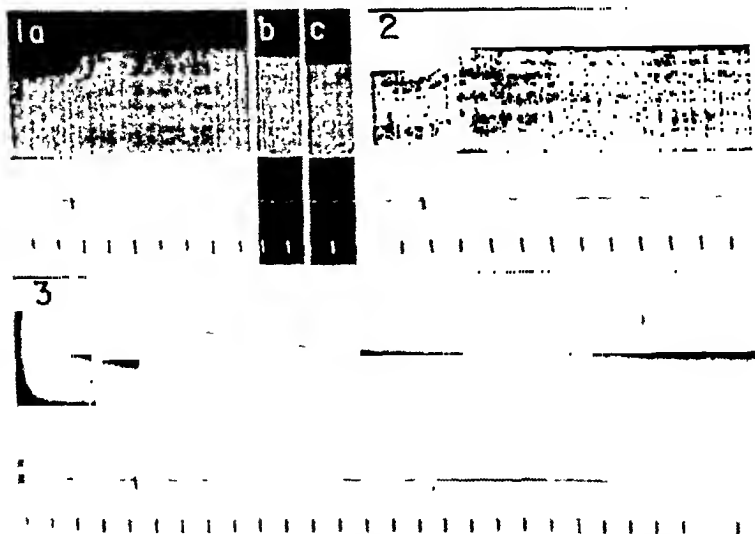


FIG. 1. INFLUENCE OF THE FREQUENCY OF STIMULATION ON THE STRENGTHENING EFFECT OF CAFFEINE
(Cats anesthetized with nembutal)

1a. Indirect excitation of the quadriceps with a frequency of 14 per second. Upper tracing, quadriceps muscle. Signal on upper line: 0.30 cc. of caffeine (in this, as in all figures, in 3.5% solution) into the femoral artery. Lower line, time every ten seconds.

1b. Three minutes after 1a.

1c. Four minutes after 1a.

FIG. 2. INDIRECT EXCITATION OF THE QUADRICEPS WITH A FREQUENCY OF FOUR PER SECOND

Upper tracing, quadriceps muscle. Upper line: signal, 0.30 cc. of caffeine into femoral artery. Lower line, time every 5 seconds.

FIG. 3. INDIRECT EXCITATION OF QUADRICEPS WITH A FREQUENCY OF 13 PER SECOND

Upper tracing, quadriceps muscle. Upper line: arrow, onset of stimulation; signal, 0.30 cc. of caffeine into femoral artery. Lower line, time every 10 seconds.

branch to the quadriceps femoris or into the abdominal aorta as described above. Others were injected intravenously (atropine, curare, and sometimes caffeine); for this the external jugular vein was isolated.

RESULTS 1. *Action of caffeine on indirect muscular stimulation.* When caffeine is injected into the abdominal aorta or into the femoral artery, in a dosage of 0.007 to 0.035 gm., while the quadriceps or soleus muscle is being stimulated through the nerve with maximum stimuli, there is always an increase

in the tension maintained by the muscle. The characteristics of this increase of tension depend on the frequency of the stimulation:

a) With stimuli of lower frequency, even as little as 10 per second (figs. 1 and 2), there is quickly noticeable an increase of the tension maintained by the muscle, a phenomenon which in some cases can last more than 4 minutes (fig. 1a, b, and c). Under these conditions the increase of tension reaches a value of 25%.

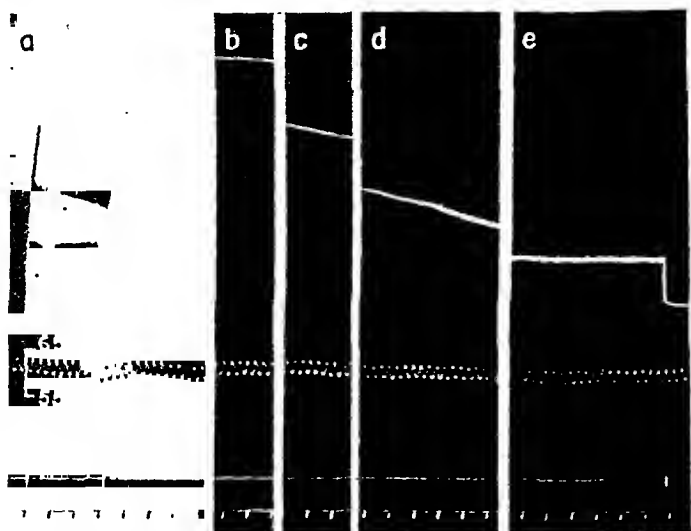


FIG. 4. INFLUENCE OF THE FREQUENCY OF STIMULATION ON THE STRENGTHENING EFFECT OF CAFFEINE: (Elliott preparation)

Upper tracing: quadriceps muscle stimulated indirectly with a frequency of 15 per second. Lower tracing: arterial blood pressure measured with a mercury manometer.

4a. Upper line: first signal (reading from left to right in this as in all figures), onset of stimulation. Lower line: time every 10 seconds.

4b. 50 seconds after 4a.

4c. 120 seconds after 4b.

4d. 90 seconds after 4c.

4e. 130 seconds after 4d. The signal on the upper line indicates the end of stimulation.

b) With greater frequencies, between 10 and 20 stimuli per second, caffeine produces the highest development of tension which we have observed (figs. 3, 4, and 5). Figure 4 shows a typical example in which the tension produced by the injection into the femoral artery of 0.012 gms. of caffeine reaches a value of 280%, and does not return to the original tension until 6 minutes later. Within these frequencies, and even more so with higher ones, caffeine tends to produce a summation of stimuli, so that if the frequency of stimulation was not sufficient to create a perfect tetanus, the action of the caffeine may bring about such a state and sustain it for some time (fig. 4 and 5).

c) When higher frequencies are used, from 20 to 250 per second, the increase

of tension developed on injection of caffeine is of little magnitude and of short duration (fig. 6, 7, and 8). As can be seen in these figures, frequencies greater than 60 per second result in a development of tension which is the smallest in

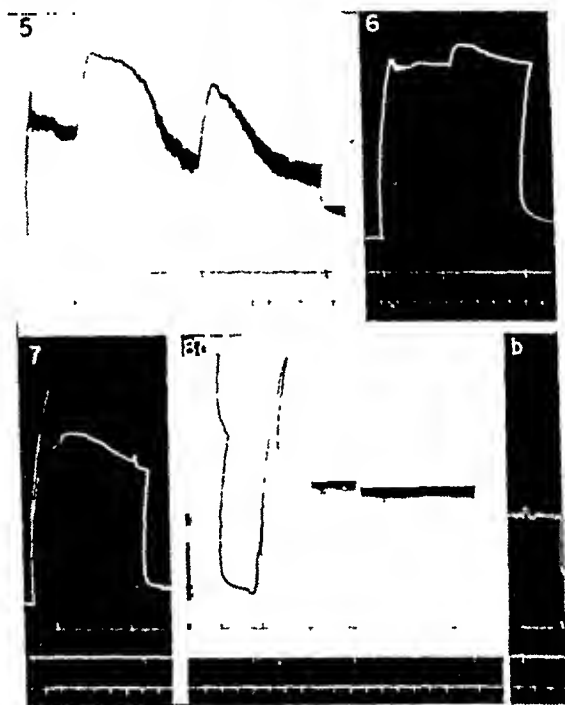


FIG. 5 INFLUENCE OF THE FREQUENCY OF STIMULATION ON THE STRENGTHENING EFFECT OF CAFFEINE
(Cats anesthetized with nembutal)

Indirect stimulation of the quadriceps with a frequency of 17 per second. Upper tracing: quadriceps muscle. Upper line: first and last signal, onset and end of stimulation, second signal, 0.30 cc. of caffeine into the femoral artery, third signal, 0.40 cc. of caffeine into femoral artery, fourth signal, 0.30 cc. of sodium benzoate, 5% (the same amount of sodium benzoate as that used as a solvent for the dose of caffeine injected at the second signal) into femoral artery. Lower line: time in minutes.

FIG. 6 INDIRECT EXCITATION OF QUADRICEPS WITH A FREQUENCY OF 23 PER SECOND. Upper tracing: quadriceps muscle. Upper line: first and third signal, onset and end of stimulation. Middle signal: 0.30 cc. caffeine into femoral artery. Lower line: time in minutes.

FIG. 7 INDIRECT EXCITATION OF QUADRICEPS WITH A FREQUENCY OF 250 PER SECOND. Upper tracing: quadriceps muscle. First line: first and second signal, injection of 0.30 cc. of caffeine into femoral artery. Second line: first and second signal, onset and end of stimulation. Third line: time in minutes.

FIG. 8 INDIRECT STIMULATION OF QUADRICEPS WITH A FREQUENCY OF 60 PER SECOND. Sa: First line: first and second signal, onset and end of stimulation, third signal, onset of stimulation. Second line: first signal, 1 cc. of caffeine into femoral artery, second signal, 0.50 cc. of caffeine into femoral artery, third signal, 0.25 cc. of caffeine into femoral artery. Third line: time in minutes.

Sb: Eight minutes after Sa. Signal on first line, end of stimulation. Signal on second line, 1 cc. of caffeine into femoral artery. Third line: time in minutes.

magnitude and of the shortest duration which we have observed, and its characteristics are the same regardless of the phase of contraction in which the muscle might be at the time (Rosenblueth and Cannon, 1940).

In the indirectly stimulated muscle the increase of tension produced by caffeine action is not parallel to the quantity of the drug injected, as is shown in figures 5 and 8. At times, however, especially when stimuli of optimum frequency are being used (10 to 20 per second), it can be seen that a greater dosage of the drug produces a greater muscular tension.

When stimuli of high frequency are being used (60 to 400 per second), injection of the drug prior to onset of stimulation does not significantly increase the height of muscular contraction, as can be seen in figure 8. Furthermore, preliminary injection of caffeine does not change to any definite degree the various phases which are observed when muscle is stimulated indirectly with high frequencies (Rosenblueth and Cannon, 1940).

II. Action of caffeine on muscle at rest. In some instances, we noted that the injection of caffeine produced a slight elevation of the base line of the muscles between low frequency stimuli (fig. 2). This led us to study the phenomenon in greater detail.

a) Action of caffeine on unstimulated muscle. When caffeine is injected intra-arterially it can at times produce a development of tension in resting muscle. We cannot at present say whether this is contraction or contracture. This phenomenon is not observed in all cases, at least not with the doses which we were using (up to 0.035 gm. of caffeine). In some instances it could only be observed with the higher doses (0.035 gm. of caffeine) as is evident in the experiment represented in figure 8. When the muscle does respond, even to the lower doses, then there can be noted a relationship between the quantity of the drug injected and the degree of tension developed, as shown in figure 9A.

The development of tension produced by caffeine can be observed not only in muscle denervated as long as 15 days previously, but in muscle which retains its innervation as well. The frequency and the characteristics of this development of tension are equal in intact and denervated muscle.

b) Action of caffeine on directly stimulated muscle. After the sectioning of a motor nerve its degeneration is so rapid and so complete that three days later stimulation of the nerve fails to produce muscular response, as indicated by a large number of experiments. (For discussion of this phenomenon see Lissak, Dempsey and Rosenblueth, 1939). For this reason, three days after nerve section stimulation of a muscle can be considered as direct. Hence, we used animals 3 to 7 days after section of either the crural or sciatic nerve, the quadriceps or soleus muscles being stimulated directly. Under these conditions there were observed results very similar to those described in Paragraph I. In fact, a 0.014 gm. dose of caffeine injected intra-arterially during muscular stimulation, whatever the frequency, always produces an increase of the height of contractions. Nevertheless, the degree and duration of the increase obtained with the caffeine are both greater when the muscle is stimulated indirectly through its nerve than when it is stimulated directly (fig. 9B, C, and D).

III. Action of caffeine on the response of muscle and ganglion stimulated by acetylcholine. A. Action on muscle. Brown, Dale and Feldberg (1936) demonstrated that the preliminary denervation of muscle increases its responsiveness to the action of acetylcholine by about 100 times, with this preparation it is possible to obtain an excellent muscular contraction with a small dose of the drug. In order to avoid large doses of acetylcholine a series of animals was subjected to denervation 5 to 15 days prior to experimentation. All of them, before receiving the acetylcholine, were administered 1 mg. of atropine per kilogram body weight intravenously, so that the muscarine effect of acetylcholine might be avoided. Once uniform responses were established in the muscles by estab-

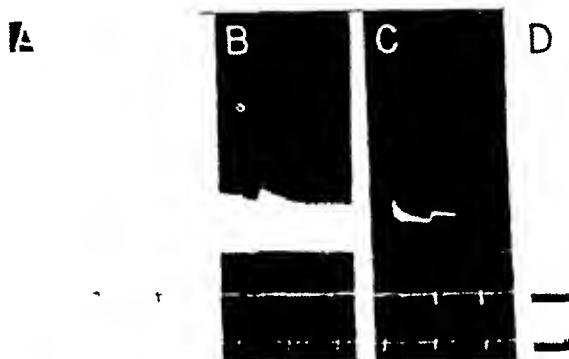


FIG 9A Direct action of caffeine on muscle. Cat anesthetized with Diil. Upper tracing soleus muscle denervated 13 days previously. Upper line first signal, intraaortic injection of 0.25 cc. of caffeine, second and third signals, 0.5 cc. and 1 cc. of caffeine intraaortically, respectively. Lower line time in minutes.

FIG 9B Action of caffeine on directly stimulated muscle. Cat anesthetized with Nembutal. Upper tracing quadriceps muscle denervated 72 hours previously and stimulated directly with a frequency of 64 per minute. Signal on upper line intraaortic injection of 0.40 cc. of caffeine. Lower line time in minutes.

FIG 9C Action of caffeine on directly stimulated muscle. Cat anesthetized with Nembutal. Upper tracing quadriceps muscle denervated 72 hours previously and stimulated directly with a frequency of 9 per second. First and second signals onset and end of stimulation, middle signal 0.40 cc. of caffeine intraaortically. Lower line time in minutes.

FIG 9D Action of caffeine on directly stimulated muscle. Cat anesthetized with Nembutal. Upper tracing quadriceps muscle denervated 72 hours previously and stimulated directly with a frequency of 50 per second. Upper line first and second signal onset and end of stimulation, middle signal 0.40 cc. of caffeine intraaortically.

lishing optimum intra-arterial doses of the drug, the caffeine was injected. A typical response is shown in figure 10. The caffeine invariably enhanced the response of denervated muscle to acetylcholine.

B. Action on the superior cervical ganglion. Employing the technique described by Roscnblueth and Cannon (1939) for the stimulation of the superior cervical ganglion by intraarterial injection of acetylcholine, it is observed that there is a double response of the motoring membrane: a primary phase due to the action of the acetylcholine on the ganglion, and a secondary due to the direct action of the acetylcholine on the smooth muscle of the motoring mem-

brane. A typical contraction of the nictitating membrane with these characteristics is produced by the injection of 40 mg. of acetylcholine dissolved in 0.20 cc. of distilled water or physiological saline. Once several contractions of uniform

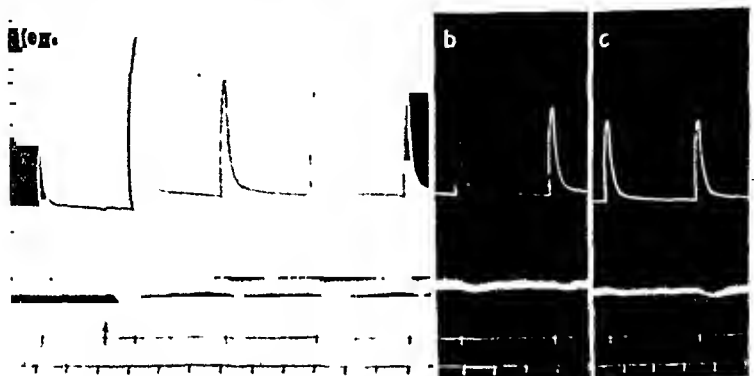


FIG. 10. ENHANCING EFFECT OF CAFFEINE ON MUSCULAR CONTRACTION INDUCED WITH ACETYLCHOLINE

Cat anesthetized with Nembutal and previously atropinized (1 mg. of atropine per kilo body weight intravenously). Upper tracing: soleus muscle denervated 14 days previously. Lower tracing: arterial blood pressure measured with a membrane manometer.

10a. Signals on the first line indicate intraaortic injections of 10 mg. of acetylcholine. The arrow indicates the moment of injection of 0.33 cc. of caffeine intraaortically. Second line: time in minutes.

10b. Ten minutes after 10a.

10c. Ten minutes after 10b.



FIG. 11 (left) Strengthening effect of caffeine on the isotonic contraction of the nictitating membrane produced by the action of acetylcholine on the superior cervical ganglion. Cat anesthetized with Dial. Upper tracing: contractions of the nictitating membrane produced by the intracranial injection of 10 mg. of acetylcholine every 4 minutes in a volume of 0.20 cc. (the external carotid artery on this side having been previously ligated). Signal on the upper line: 0.30 cc. of caffeine. Lower line: time in minutes.

degree had been obtained, the caffeine was injected. The drug consistently produced an increase of the amplitude of contraction of the muscle in the ganglionic phase; the phase ascribed to the muscle itself did not vary significantly, as can be seen in figure 11. In some cases in order to ascertain that the action was due

to the effect of caffeine on the ganglion alone, the experiments were repeated after extirpation of the superior cervical ganglion. Under these conditions caffeine seems not to have any effect on the response of the membrane to the intra-arterial injection of acetylcholine, or, if indeed it has any effect at all, it is in the form of a depressant action as can be seen in figure 12, which shows results in the same animal as those in figure 11, after the removal of the superior cervical ganglion on the side of experimentation.

IV. *Action of caffeine on the effect of prostigmine.* The quantity of prostigmine used intra-arterially (femoral artery or aorta) has been small: 50-100 mg. Nevertheless, with these concentrations, under the frequencies of stimulation used in these experiments, it was possible to observe the classical depression which prostigmine produces in the neuromuscular preparation. If under these conditions caffeine is injected, it is noted that it always augments the depressant

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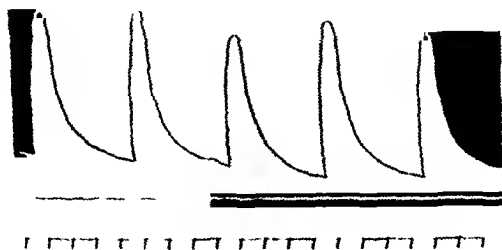


FIG. 12 (right) Same animal as in preceding figure after extirpation of superior cervical ganglion. Conditions of the experiment are the same as in Fig. 11 except that the signal on the upper line indicates the injection of 0.40 cc. of caffeine.

action of the prostigmine (fig. 13B). When an animal has received a preliminary injection of prostigmine, there is more frequently observed a certain development of muscular tension than when prostigmine has not been used: in figure 13B it is seen that the base line of muscular contractions has risen immediately following the injection of caffeine.

V. *Action of caffeine on the effect of curare.* As is evident in figures 13c and 13d, caffeine has a definite anti-curare action. This action develops quickly after the injection of the drug and attains a maximum for a given dosage of caffeine in a few seconds. For this reason a graph in which anti-curare effect is demonstrated shows a sudden break with the formation of an acute angle (figs. 13c and 13d). The duration and degree of the effect is dependent on the degree of curarization of the animal and on the dose of caffeine administered. For a uniform dose of curare, an increase in the dose of caffeine produces a corresponding increase in both the degree and duration of anti-curare effect. The dosages usually employed (0.007 to 0.035 gms. of caffeine) do not result in a complete decurarization of the preparation, and their effect does not last more than 2

minutes (fig. 13c). On the other hand, unusually large doses, as 0.21 gms. intravenously, produce a complete decurarization (fig. 13d).

VI. Effect of sodium benzoate on various experimental conditions. As has been stated above, the caffeine was dissolved in a 5% solution of sodium benzoate. It was important, therefore, to control the action which the sodium benzoate might have on the various experimental conditions described above (Sections I, II, III, IV, and V), in dosages equal to those employed in the administration of caffeine. We did not find that the sodium benzoate had any effect whatever on the various conditions described above. An example of this can be seen in Fig. 5. Hence it is possible to affirm that all the results described in this paper are due to the

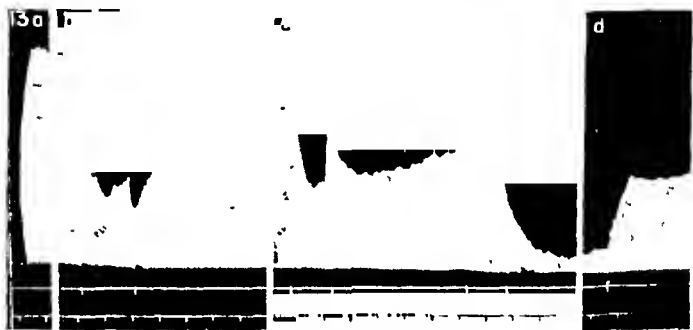


FIG. 13. STRENGTHENING ACTION OF CAFFEINE ON THE EFFECT OF PROSTIGMINE AND THE DECURARIZATION ACTION OF CAFFEINE

Cat anesthetized with Dial. Upper tracing: quadriceps muscle stimulated indirectly with a frequency of 60 per minute.

13a. Onset of stimulation. Lower line: time in minutes.

13b. Five minutes after 13a. Upper line: first signal, 75 mg. of prostigmine into femoral artery; second signal, 0.30 cc. of caffeine into femoral artery. Lower line: time in minutes.

13c. Three and one half minutes after 13b. Upper line: first signal, intravenous injection of curare; second signal, 0.30 cc. of caffeine into femoral artery; third signal, intravenous curare, fourth signal, 0.40 cc. of caffeine into femoral artery. Lower line: time in minutes.

13d. Three minutes after 13c. Upper line: 6 cc. of caffeine intravenously at the signal. Lower line, time in minutes.

action of caffeine and not to any effect of sodium benzoate which was used as a solvent for the caffeine.

DISCUSSION. From the above described results it is possible to deduce that caffeine acts on the neuromuscular preparation through a double mechanism: first, by a direct action on the muscle, and second, by a direct action on the neuromuscular junction. The study of the latter action is the one which was of greater interest to us.

It can be said that the strengthening effect of caffeine on indirect muscular contraction (see Results, Section I) and on the response of muscle and the ganglion stimulated by acetylcholine (see Results, Section III), is not due to the general vasodilatory effect of the drug, because the hypotension produced by the drug when injected intra-arterially in the dosages here employed is of very slight degree (figs. 4 and 10). Nevertheless, it could be claimed that the local vaso-

dilatation which the drug produces could improve the neuromuscular circulatory conditions and that this in turn could be the cause of the strengthening effect of the drug, and not a direct action of the caffeine on the neuromuscular junction. However, other drugs which produce vasodilatation do not give this effect. Furthermore, the effect of the caffeine maintains a special relationship to the frequency of stimulation, a fact which without doubt points to a neuromuscular mechanism and not to a vascular one. The fact that the effect of caffeine is added to the depressant effect of prostigmine (see Results, Section IV), could not be explained as a result of vasodilatation.

It is clear that caffeine acts directly on the neuromuscular junction and on the synapses of the ganglion. Rosenblueth and Morison (1937) state that acetylcholine has a double effect on muscle, one excitatory and the other depressant. So as to have an excitatory effect the concentration of acetylcholine must surpass a certain level, the excitatory threshold which is lower than, and bears a certain relation to, the paralytic threshold; that is, the level of concentration of acetylcholine necessary to produce a depressant effect. In view of these points, one may well suppose that caffeine has the property of lowering the excitatory threshold of acetylcholine, whether we speak of that liberated by stimulation of the nerve or that injected experimentally. The decurarization effect and the strengthening of prostigmine by caffeine are further arguments in favor of the concept.

The pharmacological effect of caffeine on the neuromuscular junction is quite similar to that of eserine or of prostigmine. In fact, caffeine lowers the excitatory threshold of acetylcholine; eserine and prostigmine also lower it (Rosenblueth and Morison, 1937), the three drugs thus giving the noted effects on acetylcholine and curarized preparations. Nevertheless, there are certain differences: caffeine does not inhibit the blood choline esterase in man in therapeutic doses (0.4 to 0.5 gm., Schmets, 1943) despite the fact that Bernheim and Bernheim (1936) maintain that the drug inhibits choline esterase in the central nervous system, whereas the depressant effect of eserine and prostigmine on choline esterase is classical. This fact, though disputed, may contribute to the explanation of caffeine's effect being of less duration and intensity than that of the other substances. There is, however, one major difference entering into this analogy: eserine and prostigmine only enhance indirect muscular contraction when stimulation is applied at a very low frequency, for example, one every 10 seconds (Brown, Dale and Feldberg, 1936; Rosenblueth and Morison, 1937); under other conditions these drugs only produce depression. In contrast, caffeine enhances the tension developed by the muscle regardless of the frequency of stimulation, whether direct or indirect. This discrepancy may be ascribed in part to an action of these drugs on muscle described elsewhere (Rosenblueth, Lundley, and Morison, 1936) and differing from that described in this paper: Section II of Results.

Our observations again demonstrate the similarity between the neuromuscular junction and the superior cervical ganglion since in both caffeine enhances the response produced by acetylcholine (Cannon and Rosenblueth, 1937).

SUMMARY

In cats anesthetized with Dial or Nembutal, or in cats which have undergone decerebration or removal of the central nervous system, studies have been made on the effect of caffeine injected intra-arterially in doses of 0.007 to 0.035 gms. on: a) the contractions of skeletal muscles; b) the neuromuscular synaptic mechanism; c) the superior cervical ganglion stimulated by acetylcholine.

Caffeine produces an increase in the tension developed by muscle which is being stimulated indirectly (figs. 1, 2, 3, 4, 5, 6, 7, 8 and Results, Section I).

Caffeine can produce a development of tension in normal muscle (fig. 8) as well as in denervated muscle (fig. 9A). When denervated muscle is stimulated directly, caffeine can also increase the amplitude of contractions (fig. 9B, C, and D, and Results, Section II).

Caffeine is able to augment not only the amplitude of contractions of a muscle stimulated by means of acetylcholine, but also those of the nictitating membrane similarly stimulated by the latter drug (fig. 10 and 11). The enhancing action of caffeine on the contractions of the nictitating membrane stimulated by acetylcholine is due to the effect the caffeine exerts upon the ganglion (figs. 11 and 12 and Results, Section III).

Caffeine is a decararizing drug (fig. 13) and augments the action of prostigmine (fig. 13; Results, Sections IV and V).

The mechanism of the action of caffeine on the neuromuscular junction is discussed and the conclusion is reached that it lowers the excitatory threshold of acetylcholine. The similarities and differences between the pharmacological action on the neuromuscular junction of caffeine on the one hand and of eserine and prostigmine on the other, is discussed.

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EXPERIMENTAL AND CLINICAL STUDIES ON THE ACTION OF HIGH DOSES OF HYKINONE AND OTHER MENADIONE DERIVATIVES

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It is necessary to determine whether large amounts of antihemorrhagic quinones given to promote prothrombin synthesis will exert toxic effects. Generally small doses, 2-4 mg. in magnitude, have been found adequate to correct prothrombinopenic states as they occur clinically. More recent studies, however, have disclosed the need for larger doses in the following:

1. As an antidote to dicumarol excess (1-4)
2. As a prophylactic against salicylate-induced prothrombinopenia (5-8)
3. As a liver function test (9)

In 1942 Ansbacher, Corwin, and Thomas (10) studied the toxicity of menadione, menadiol, and their esters. Their work established that in animals treated with these compounds or their derivatives the pathological changes involved primarily the red cells, the chief manifestations of toxicity being hemolytic anemia. The 50 per cent lethal dose of menadione given subcutaneously was enormous: 138 mg. per kilo. Definite anemia was observed in cats and dogs after 5-7 injections of 20 to 25 mg. per kilo of menadione.

We have investigated in animals and in man the signs of acute and chronic toxicity following the administration of Hykinone.¹ In mice, rabbits, and dogs excessively high and lethal doses of Hykinone were used; in man therapeutically active doses were given in the course of diagnosis or treatment of various clinical disorders. The drug was administered orally and intravenously.

ANIMAL EXPERIMENTS Extensive studies were conducted in mice, rabbits, and dogs to determine the acute toxicity of Hykinone. In addition, several series of experiments were carried out in dogs with high but sublethal doses administered daily intravenously for varying periods of time. In order to conserve space, all these data are presented here in a very condensed form.

Acute toxicity: 26 mice were given 1 per cent solution of Hykinone intravenously. The approximate LD 50 was found to be 250 mg. per kilo. Death

¹ Hykinone is the registered trademark of the Abbott Laboratories for their original Menadione Bisulfite Anhydrous. Recently the firm has changed their use of this mark to refer to the Menadione Bisulfite of the U S P which contains 3 molecules of water. Hence, all doses of Hykinone referred to in this paper must be multiplied by 1.2 to convert them to the present Hykinone (Menadione Bisulfite, U S P).

occurred 10 to 15 minutes after injection due to respiratory paralysis, and was preceded by convulsions, lacrimation, and exophthalmos.

A 5 per cent solution of Hykinone was injected intravenously into 25 rabbits. The LD 50 was found to be approximately 120 mg. per kilo. Most of the animals died in convulsions which appeared 15 to 30 minutes after the administration. Dilatation of the heart was the only pathological finding. Discoloration of the blood was not observed. Some of the animals died 1 or 2 days later, although no significant symptoms were observed.

Seventeen dogs were given 25 to 150 mg. per kilo Hykinone intravenously as 5 per cent solution. Only one dog died after 100 mg./kg. but 3 out of 3 died after 150 mg./kg. Salivation, vomiting, and restlessness appeared a few minutes after the injection. Death invariably followed within 1 to 24 hours. A gray-bluish cyanosis arose about 10 minutes after the injection and the blood drawn at this time presented a dark and sometimes brownish appearance. This was noted occasionally after doses of 50 mg./kg. were injected and regularly after larger quantities. The presence of methemoglobin was suspected and in 2 dogs which received 100 mg./kg. intravenously, samples of blood drawn before the administration of Hykinone and at intervals of 15 to 50 minutes thereafter and examined spectrophotometrically, showed the presence of methemoglobin 15 minutes after the injection. The quantity of methemoglobin commenced to decrease after 30 minutes so that at the end of 45 minutes this pigment could no longer be demonstrated and at the same time the external indications of cyanosis began to disappear.

Three dogs were injected with high but sublethal doses of Hykinone repeatedly by the administration of 25 mg./kg. intravenously twice daily for three days and then 50 mg./kg. once daily for two days. On the sixth day 80 mg./kg. were injected, which was followed by death of the animals. Prothrombin times of the diluted (12.5%) plasma determined by the single stage method of Quick varied between 27 and 28 seconds before the injection and became reduced to 13, 16, 19 seconds respectively on the third day of the experiment. Another group of three dogs was subjected to the same procedure and showed an almost identical course. The decalcified plasma obtained from these animals clotted promptly upon the addition of merely the thromboplastin before recalcification was executed. All six dogs showed the dark discoloration of the liver, cloudy swelling and, in a few instances, early necrosis. All these animals developed severe anemia, as described in the following studies on chronic toxicity.

CHRONIC TOXICITY. Chronic toxic effects were induced by daily administration of 15 to 40 mg./kg. Hykinone intravenously for a period of 15 days. Extensive data on all the animals were collected. No particular purpose would be served by presenting all these findings; hence we have reproduced in table 1 the protocols of four dogs which gave typical responses.

Differential blood counts were made in all animals but revealed no significant changes, and are omitted. All of this group of dogs survived the experiments. Dogs I and II were sacrificed to obtain autopsy material. No important change in weight occurred. Urine findings were negative with one exception, in which

albuminuria was noted after the last injection. Urobilinogen was moderately increased in 3 out of 4 cases, and a few red blood cells appeared in 3 of the animals' specimens. During the height of the induced anemia, normoblasts, not exceeding 6 per cent, were detected in the blood of some of the dogs except in one instance in which the frequency of these cells was 32 per cent. Promptly following the withdrawal of the Hykinone the normoblasts disappeared.

TABLE 1

Effect of daily intravenous injections of high doses of hykinone in dogs

	DOG	CONTROL	INJECTION PERIODS			DAYS AFTER LAST INJECTION	
			3 days	6 days	15 days	13	23
Hb gm. %	1	11.0		10.0	5.0		
	2	14.0		10.6	5.5		
	3	14.0			7.0	14.0	14.0
	4	14.0			9.7	12.6	16.0
R.B.C. Mil- lions	1	3.88		4.28	1.98		
	2	6.51		4.72	2.20		
	3	5.95			2.61	6.51	6.08
	4	5.70			4.12	4.94	6.78
W B C	1	15050		25850	17300		
	2	17200		26750	25400		
	3	8850			9850	7600	9650
	4	10750			17250	16400	21150
Prothrombin times in sec- onds (12.5% plasma)	1	18.5	17.0	19.5	16.5		
	2	21.0	18.5	20.0	16.5		
	3	18.0			14.0*	19.0	26.0
	4	17.0			19.0*	21.0	21.0
Plasma pro- tein gm %	1	6.60			5.54		
	2	6.97			6.70		
	3	5.41				5.72	5.75
	4	5.34				5.31	6.53

* Taken after the 10th injection

Remarks Dog 1 received daily 40 mg/kg Hykinone intravenously

Dog 2 received daily 25 mg/kg Hykinone intravenously

Dog 3 received daily 15 mg/kg Hykinone intravenously

Dog 4 received daily 15 mg/kg Hykinone intravenously

In dog I, immediately after the animal was sacrificed by chloroform, remarkable acceleration of the clotting of the blood was observed. There were patches of yellowish discoloration throughout the body cavities, but no other gross findings. On histological examination extensive fatty degeneration and cloudy swelling of the liver was seen. Degenerative changes were noted in the kidney, with cast formation and impairment of nuclear staining. In dog II in addition

to the degenerative changes noted above, some irregularities of the cortical cells of the adrenals were found.

A comparative study was made using 2 methyl-1,4 naphthahydroquinone diphosphoric acid ester tetrasodium salt (synkayvite).² Two dogs were injected with 15 mg./kg. of this drug daily for 15 days. The changes in the blood, including the prothrombin time, were similar to those found after Hykinone.

DISCUSSION OF RESULTS. The low degree of acute toxicity revealed in the experiments in mice, rabbits, and dogs leads us to conclude that the highest dosage levels of antihemorrhagic quinone used in man to counteract dicumarol-induced prothrombinopenia are relatively harmless. Smith, Ivy, and Foster (11) arrived at similar conclusions in respect to the administration of Hykinone and Synkayvite in animals. They also noted anemia after repeated injections of large doses of Synkayvite. We observed a constant depression of hemoglobin and erythrocytes with concomitant appearance of normoblasts after repeated large doses of Hykinone. These effects, however, subsided promptly with restoration of the normal blood picture upon the withdrawal of the medication. Ansbacher and his co-workers (10) favor the view that there is injury to the circulating red cells; we found no evidence of hemolysis *in vivo*.

The leukocytes revealed no constant alteration during or after the injections of Hykinone nor did the plasma proteins yield any significant depression even at the height of the anemia. Accelerated blood clotting as evidenced by marked hyperprothrombinemia was noted in some of the dogs after large doses of Hykinone. In the subacute toxicity studies the prothrombin time of the diluted (12.5%) plasma was reduced to approximately one-half the original value in a few days. Similarly but less pronounced was the response noted in the chronic toxicity series.

While this work was in progress, Field and Link (21) reported an increase of the prothrombin time in experimental animals after a single large dose of menandione.

We wish to emphasize two facts:

1. That only by the use of diluted plasma is it possible to detect the hyperprothrombinemia
2. The prothrombin time becomes extended after protracted treatments with Hykinone.

This phenomenon might be explained, at least in part, by the liver damage noted at autopsy. Its counterpart (prolongation of the prothrombin time) has been observed by us in man in cases of hepatic disease in which Hykinone was administered at high dosage levels. It has proved especially significant in cases of suspected liver disease with initial normal prothrombin levels and a procedure embodying these features has been utilized to detect subclinical liver damage (9).

Urobilinogen appeared in the urine. This may have been due either to a direct toxic action of these high doses upon the liver or to the severe anemia. It is noteworthy that transitory methemoglobin was detected exclusively in dogs, and after very high doses of Hykinone.

² Supplied through the courtesy of Dr. Foster of the Hoffman La Roche Company.

OBSERVATIONS IN MAN. Studies were made in a group of cases including the following: portal (Lacnec's) cirrhosis, hemachromatosis, acute rheumatic endocarditis, acute arsenical hepatitis, macrocytic (primary) anemia, "idiopathic hypoprothrombinemia," non-tropical sprue, nutritional (microcytic) anemia, arthritis, and arthralgia. The respective quantity and mode of application of the antihemorrhagic quinone used in each case will be indicated later in the paper. Since the work in animals established the toxic effect to consist chiefly in the depression of the hemoglobin and erythrocyte content of the blood, we sought to determine to what extent large therapeutic doses of synthetic vitamin K induced anemia or aggravated such condition when it was already existing. In addition serial prothrombin estimations were made in each case during the entire period of observation. Blood counts were made generally before, during, and after the course of treatment.

The method used for the estimation of the prothrombin time is identical with that described in detail in previous papers of this series (12-14). It utilizes the principle of diluted plasma prothrombin time, by virtue of which the sensitivity and reliability of the procedure is greatly augmented. Estimation of the diluted (12.5%) plasma prothrombin time was made almost daily, at least five times each week. The normal standard for man is 39.5 seconds (standard deviation ± 2.5). The thromboplastin was prepared from desiccated fresh rabbit lung.

In twelve cases the synthetic vitamin K was given perorally in conjunction with salicylate therapy. These cases had exhibited slight or moderate² prolongation of the prothrombin time after having received salicylate alone. Acetyl salicylic acid (1-2 g. daily) was used exclusively in this series.

After the course of salicylate administration, the same dose was continued and in addition antihemorrhagic quinone in proportion of 1 mg. to 15 mg. to each gram of aspirin.³ The duration of the combined therapy was in eight cases between 21 and 30 days; in single instances it was 6, 7, 11, and 14 weeks respectively. All but two cases revealed gradual reduction of the prothrombin time to normal. In these two instances slight prothrombinopenia continued despite the added synthetic vitamin K. One of the cases showed fluctuations in prothrombin time, occasionally, yielding slightly prolonged prothrombin times for short intervals, but for the most of the time showing normal figures. In two cases the diluted (12.5%) plasma prothrombin time became reduced to hyperprothrombinemic levels of 27 and 30 seconds respectively. Neither the erythrocyte count nor the hemoglobin content of the blood became depressed in any of the twelve cases. The findings in the case which received combined medication for the longest period and to which more than 2 g. of the antihemorrhagic

² Slight—diluted plasma prothrombin time less than $1\frac{1}{2}$ times normal, moderate—between $1\frac{1}{2}$ and 2 times normal (15)

³ Three preparations were used in this study: 1 Hykinone—1 mg. to each 1 g. aspirin (supplied by Abbott Laboratories, North Chicago, Illinois)

2 Namarquin (Menadione bisulfite) 2 mg. to each 1 g. aspirin (supplied by Dr. S. N. Gordon, Eudon Products, Inc.)

3 Synkavite 15 mg. to each 1 g. aspirin (supplied by Dr. Leo A. Pirk, Hoffman-La Roche Company)

quinone had been given (more than half of this was Synkayvite) were as shown in table 2.

Five cases of acute rheumatic endocarditis, each receiving salicylate (sodium salicylate or acetyl salicylic acid) and each showing prothrombinopenia were given Hykinone intravenously 5 mg. daily for four days one week and double the amount on the same number of days the succeeding week, totalling 60 mg. in each instance. In two of the patients only partial correction of the prothrombinopenia occurred, whilst of the remaining three, two revealed normal prothrombin time and one hyperprothrombinemia, after the Hykinone. The other blood constituents were unchanged after the Hykinone therapy in all five cases. It is noteworthy that the erythrocyte sedimentation rate was unaffected by Hykinone treatment. In one case it became accelerated because of aggravation of the rheumatic condition; in another case it continued increased throughout the period of observation because the activity within the heart was unabated. In the others it was normal before Hykinone was given and remained unchanged after the experiment.

Since the liver is the site of prothrombin synthesis, in the event of prothrombinopenia, the response to vitamin K may be used as a test of the liver function.

TABLE 2

DAYS OF MEDICATION	Hb %	R B.C.	W.B.C.	DILUTED (12.5%) PLASMA PRO- THROMBIN TIME
				seconds
Initial figures	98	5,160,000	11,200	51.0
98	95	5,220,000	12,000	37.5

In another communication (9) we have reported investigations in animals and man establishing the high degree of sensitivity and reliability of such a test. The procedure includes daily parenteral administration of about 20 mg. of anti-hemorrhagic quinone for 3 to 6 days. In the presence of liver disease an established prothrombinopenia is not susceptible to correction by high dosage of menadione bisulfite and in the event of normal initial prothrombin time, the repeated injection of Hykinone in the amounts stated, may prolong the prothrombin time beyond normal for one or two days. The initial series studied included 18 cases of Laennec's type of cirrhosis and two of hemachromatosis and other diseases implicating the liver in the general disturbance.⁵ Of the group one case of portal cirrhosis exhibited a temporary anemia which promptly returned to normal after Hykinone was withdrawn (see Chart 1).

Particularly significant in respect to detection of possible toxicity of anti-

⁵ The cases of hemachromatosis included in the series revealed initially severe anemia as part of the basic disturbance. The prothrombin curves after Hykinone were typical of hepatic disease, the presence of which was revealed at autopsy. This is being reported elsewhere by others (18). It was not possible to relate the severe anemia to Hykinone therapy.

hemorrhagic quinone was the behavior of the blood in cases of established anemia, of both macrocytic (primary) and microcytic (nutritional) types. One case of the former which had been in a state of remission for about one year, induced by liver therapy, was given 20 mg. Hykinone intravenously daily for six days. The hemoglobin and erythrocyte count were 112 per cent and 4.9 million before the treatment and 110 per cent and 5.3 million respectively after it. Similar findings were obtained in a case of nutritional anemia and a case of non-tropical sprue (see Charts 2 and 3). One case of chronic lymphatic leukemia showed no change in the blood picture after 120 mg. Hykinone given intravenously over a

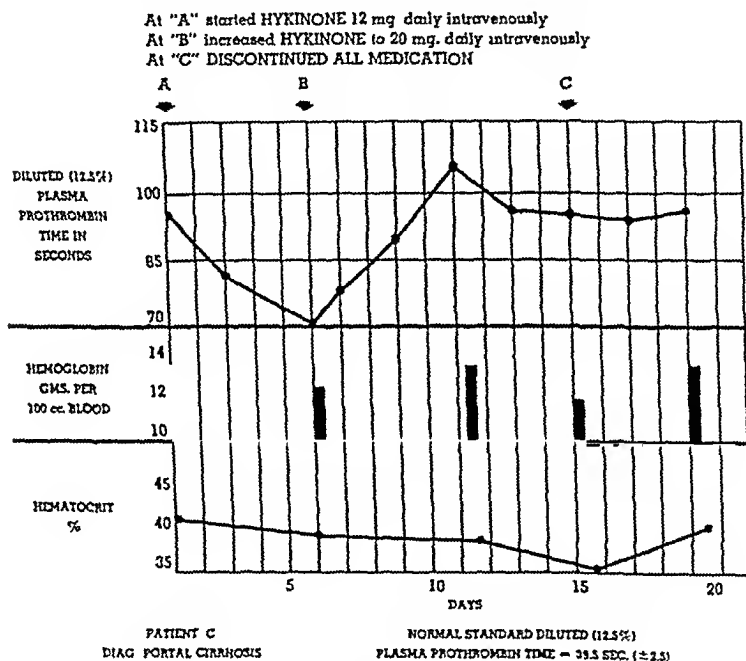


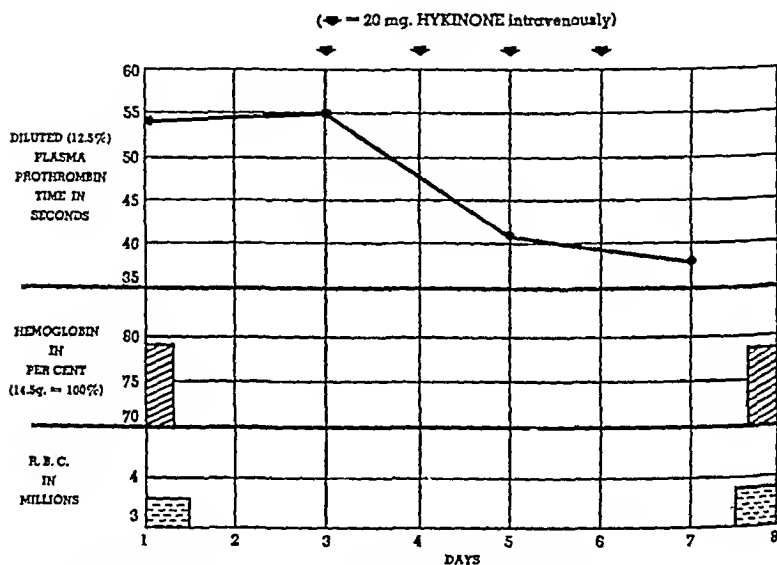
CHART 1

period of one week. A specimen of this patient's blood obtained a few hours after the last injection of Hykinone was examined spectroscopically and no methemoglobin was detected.

A 27 year old married colored female, presenting a picture corresponding to that described as "idiopathic hypoprothrombinemia" (17), was given large doses of Hykinone to correct the coagulation defect and possibly control uterine bleeding, oozing of the gums after slight trauma, ecchymoses of the skin after trivial injury, and frequent nose-bleeds. Huge doses of ascorbic acid did not alter the condition nor did the injection of 190 mg. of Hykinone reduce the consistently prolonged prothrombin time during this interval and a definite

anemia occurred, presumably as a consequence of the hemorrhage. Attention is drawn to the fact that the character of the prothrombin curve after the Hykinone was identical with that seen in cases of liver disease and described above (9) (see Chart 4).

DISCUSSION. Consideration of the toxicity of the antihemorrhagic quinones should be of one of several orders depending upon the dosage level used. When massive quantities are administered adequate to induce acute lethal effects, or 50 per cent of this amount, the toxic action of the quinone radical per se appears irrespective of prothrombin stimulation. Under such a condition extensive visceral damage occurs before the vitamin K-like activity appears. It is akin



PATIENT: J. B. AGE: 70
DIAG: NUTRITIONAL ANEMIA

NORMAL STANDARD DILUTED (12.5%)
PLASMA PROTHROMBIN TIME = 39.5 SEC. (± 2.5)

CHART 2

to the acute toxicity observed after huge doses of dicumarol before the prothrombinopenia can be produced (19).

The animal experiments described above demonstrate the occurrence of profound toxicity after dosages of about 100-150 mg./kilo. These dosage levels proved lethal to dogs. Methemoglobin formation and, at autopsy, acute degenerative changes especially in the liver were observed.

A second order of toxicity is that induced by sublethal doses in animals and in clinical application where more than the usual therapeutic amounts are needed. The clinical uses of these larger quantities are:

1. To counteract excessive dicumarol-induced prothrombinopenia

2. To prevent prothrombin depletion by salicylates

3. As a liver function test.

1. Prothrombinopenia consequent to dicumarol administration is susceptible to correction by antihemorrhagic quinones. The quantity necessary is 60-100 mg. intravenously. Only a few injections are required, depending upon the demands of the particular case. In our investigations comparable amounts of Hykinone even when administered to patients with preexisting hematological disorders, were followed by no alteration in the blood picture nor by any aggravation in the prevailing hematological disease.

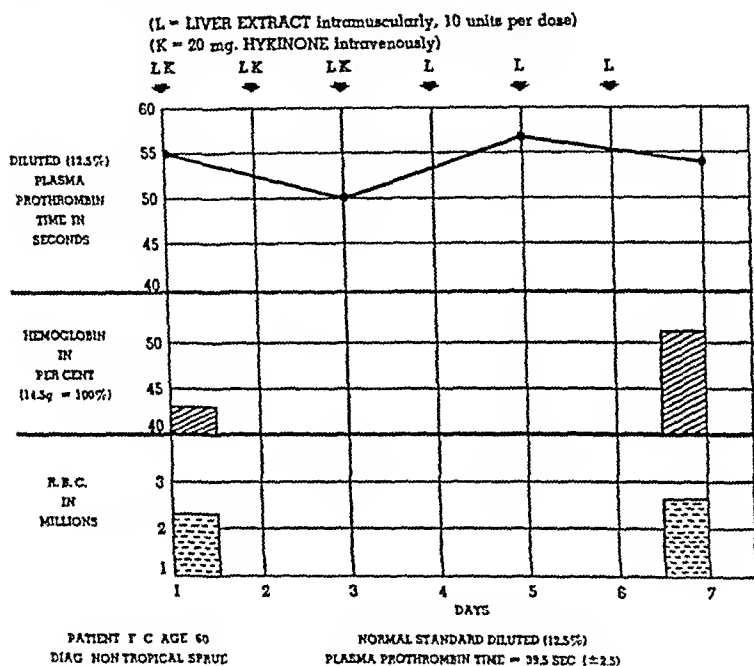


CHART 3

2. Salicylates are prothrombinopenia-inducing agents. It has been recommended that, where these drugs are administered and the prothrombin time found prolonged, antihemorrhagic quinone be added to the therapy. Our findings indicate that combined oral aspirin and vitamin K-like quinone continued for as long as 14 weeks was unattended by any toxic effect upon the erythrocyte and hemoglobin content of the blood.

Similarly when large doses of salicylates had been administered in cases of active rheumatic endocarditis and Hykinone was added to counteract the prothrombinopenia, no toxic effect upon the blood was detected. It is noteworthy that the erythrocyte sedimentation rate was in each case compatible with the clinical condition and not altered by antihemorrhagic quinone.

It was possible to examine at post-mortem the tissues of one of the cases which had been given large doses of salicylates in the treatment of active rheumatic endocarditis. Hykinone had failed to correct the prothrombinopenia; in fact, the prothrombin time became further extended after the menadione bisulfite had been administered. The only significant pathology, other than in the heart, was found in the liver. The gross appearance of the organ was typically "nutmeg" and histologically, a picture characteristic of advanced cirrhosis was noted.

3. The detection of subclinical liver disease appears to be possible by determination of the prothrombin time of the diluted (12.5%) plasma and the response to relatively large doses of Hykinone. Persistent prothrombinopenia despite the

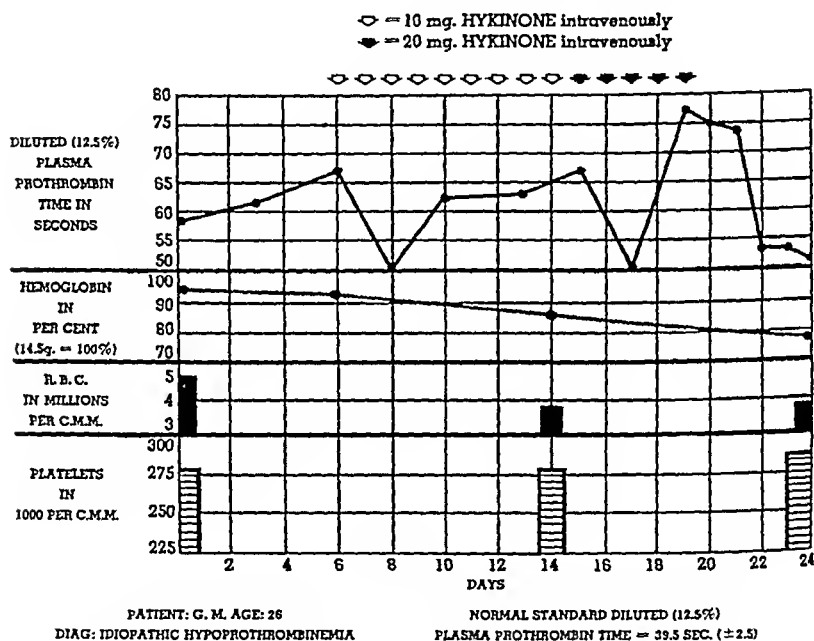


CHART 4

administration intravenously of the vitamin K-like substance has been found to occur in the presence of disturbed liver function. We find that in the event of preexisting normal prothrombin time, the injection of Hykinone repeated on several successive days is followed by temporary prolongation of the prothrombin time beyond the limits of normalcy, if liver damage is present. Actually, this response of extended prothrombinopenia may be very marked when the initial prothrombin level is below normal. Because of this paradoxical behavior the use of vitamin K-like quinones should be avoided in cases of liver disease especially where hemorrhage is imminent or has recently occurred.

We have observed evidences of toxicity very rarely when relatively large

quantities of Hykinone have been used. Although several cases with advanced cirrhosis of the liver were included in the series, in only one was anemia observed, and in this case the blood picture returned to normal promptly after the quinone derivative was withdrawn. In other instances in which specific therapy was being administered to correct established anemia, the Hykinone did not interfere with the favorable response to the hematopoietic agents.

A single case which presented the clinical characteristics of "idiopathic hypoprothrombinemia" showed a prothrombin response to high dosage levels of Hykinone identical with that of liver disease; i.e., a temporary aggravation of the prothrombinopenia and, by virtue of it, increased tendency to hemorrhage.

Other toxic effects exerted by quinones are suggested by their capacity to depress artificially induced hypertension (20). This opens an interesting field in the possible therapeutic applications of these compounds to the control of some types of hypertension.⁶

CONCLUSIONS

The lethal agent in menadione bisulfite addition product (Hykinone) is the quinone radical. It produces its effect acutely and irrespective of prothrombin synthesis.

Repeated injections of dosages greatly in excess of those generally used for correction of prothrombinopenia in man but sublethal, produce depression of the erythrocyte and hemoglobin content of the blood which promptly reverts to normal upon withdrawal of the drug.

In man doses approximately ten times as great as those generally recommended for therapeutic use, given daily for a period of one week, induce no significant changes in the blood.

In normal subjects large doses of Hykinone can produce temporary hyperprothrombinemia; in cases of liver disease similar amounts of menadione bisulfite extends the prothrombin time.

A liver function test of high sensitivity is established by the effect of anti-hemorrhagic quinones in the presence of hepatic disease. By this means it is possible to detect liver disturbances in subclinical stages and when the existing prothrombin level is normal.

The long continued administration of synthetic vitamin K preparations combined with aspirin is accompanied by no alteration in the hemoglobin and erythrocyte content of the blood.

When Hykinone is administered in cases of active rheumatic endocarditis to counteract the prothrombinopenia caused by salicylate medication, the blood remains free of toxic changes.

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⁶ The capacity of certain quinones at different dosage levels to reduce various types of clinical hypertension is being investigated by one of us (S S) and his associates, and will be reported separately in due time.

It was possible to examine at post-mortem the tissues of one of the cases which had been given large doses of salicylates in the treatment of active rheumatic endocarditis. Hykinone had failed to correct the prothrombinopenia; in fact, the prothrombin time became further extended after the menadione bisulfite had been administered. The only significant pathology, other than in the heart, was found in the liver. The gross appearance of the organ was typically "nutmeg" and histologically, a picture characteristic of advanced cirrhosis was noted.

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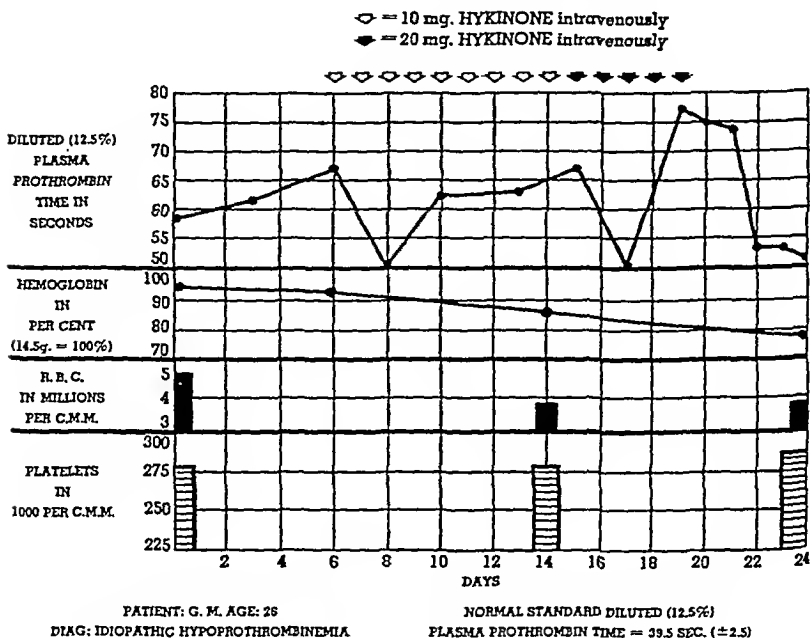


CHART 4

administration intravenously of the vitamin K-like substance has been found to occur in the presence of disturbed liver function. We find that in the event of preexisting normal prothrombin time, the injection of Hykinone repeated on several successive days is followed by temporary prolongation of the prothrombin time beyond the limits of normalcy, if liver damage is present. Actually, this response of extended prothrombinopenia may be very marked when the initial prothrombin level is below normal. Because of this paradoxical behavior the use of vitamin K-like quinones should be avoided in cases of liver disease especially where hemorrhage is imminent or has recently occurred.

We have observed evidences of toxicity very rarely when relatively large

STUDIES ON THE GENERAL PHARMACOLOGY OF DIBUTOLINE

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A series of surface-active choline derivatives has recently been synthesized by one of us¹ in collaboration with Dr. Swan of the Department of Ophthalmology. One of these new synthetics, namely: dimethyl-ethyl- β -hydroxyethylammonium sulfate di-n-butyl carbamate, called dibutoline, was found to be the most effective compound of the series in producing mydriasis and cycloplegia (1, 2). The ocular pharmacology and clinical uses of dibutoline have been reported by Swan and White (3). These authors made no detailed studies on the systemic action of this compound, which was considered of sufficient importance to warrant more exhaustive experiments on its general pharmacology. Other than the toxicological studies, the experiments have been directed chiefly to a study of the anti-spasmodic activity of dibutoline and such other experiments as might give some insight into its mode of action.

TOXICITY The toxicity of dibutoline was studied in albino rats following intraperitoneal injection and administration by stomach tube. Injections were given as one per cent solutions in physiological saline in doses calculated as milligrams per kilogram. Fifty rats weighing approximately one hundred seventy grams each were employed and were observed for twenty-four hours for fatal outcome. The LD_{50} was found to be 22 mgm./kgm. for intraperitoneal injection. When as little as 15 mgm./kgm. were given by this method the rats exhibited labored respiration, and when the dose was increased, respiratory difficulties became more severe. Death was preceded by tremors and convulsions. After oral administration of as much as 500 mgm./kgm. of the drug no symptoms of any kind were observed.

ANTISPASMODIC ACTIVITY ON ISOLATED MUSCLE. Intestinal: Isolated smooth muscle segments of rabbit gut were employed in oxygenated Tyrode's solution by the Magnus method (4). Drugs were added directly to the bath. Five rabbits were used in carrying out the eighty experiments on this phase of the work. Direct effects of dibutoline in the absence of previously induced stimulation were observed, as well as the ability of the drug to antagonize a typical musculo-stimulant drug such as barium chloride in a strength of 1:25,000 and a typical neurotropic drug like mecholyl at 1:12,500,000 concentration. In figure 1 are shown the direct effects of dibutoline in varying concentrations on isolated strips of rabbit gut. In each instance there was a slight loss of tonus accompanied by little interference with the contractions of the muscle.

The stimulation induced by mecholyl in a concentration of 1:12,500,000 is shown in figure 2, A, followed by a demonstration of the antagonistic effect of dibutoline in a concentration of 1:400,000. The third addition of mecholyl, after

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dibutoline, caused only a barely perceptible stimulation of the muscle, showing that the dibutoline present had blocked in some manner the stimulating action of mecholyl. Although dibutoline in 1:400,000 solution was found to be the most effective concentration for consistence in returning the gut strips to normal after stimulation by mecholyl, concentrations as low as 1:2,000,000 were also effective

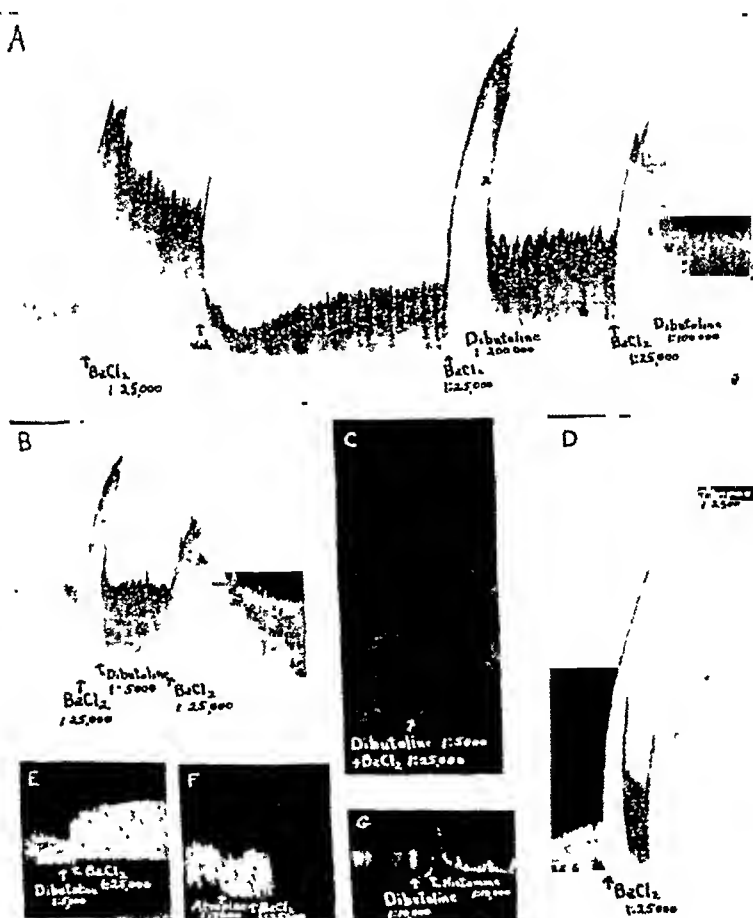


FIG. 3

(figure 2, B). This lower concentration compared favorably with the action of 1:5,000,000 atropine (figure 2, D). When dibutoline was given simultaneously with mecholyl and atropine was given simultaneously with or before mecholyl, the responses obtained (figures 2, E, F) were similar to those shown with dibutoline alone in figure 1. Although dibutoline was effective in overcoming or blocking

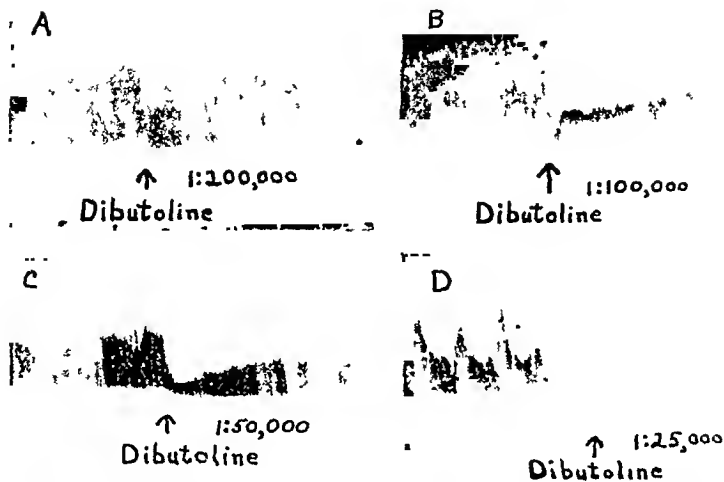


FIG. 1

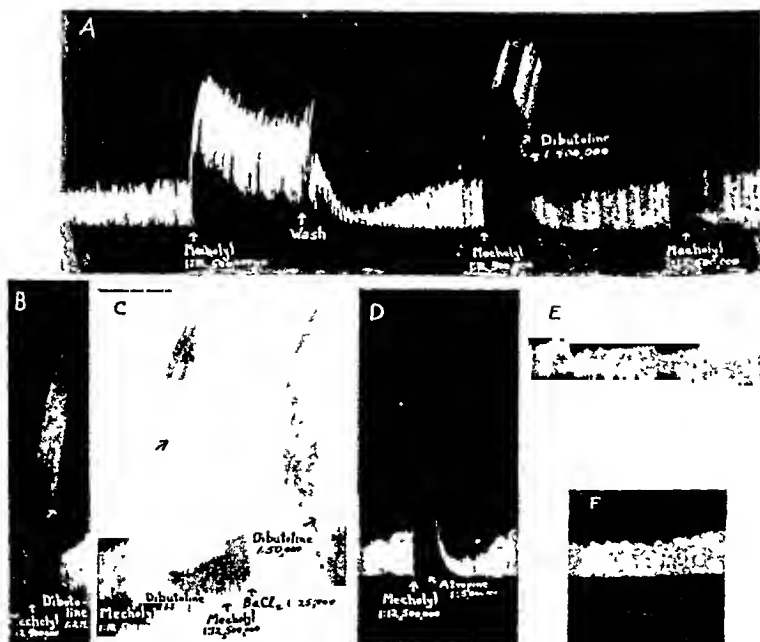


FIG. 2

One dog was prepared with a Thiry-Vella loop and a colon fistula and on six different days tracings were made of the intestinal movements as above. A record of the normal contractions of the small intestine and colon in the unanesthetized dog was made for comparative purposes and is shown in figure 5, C.

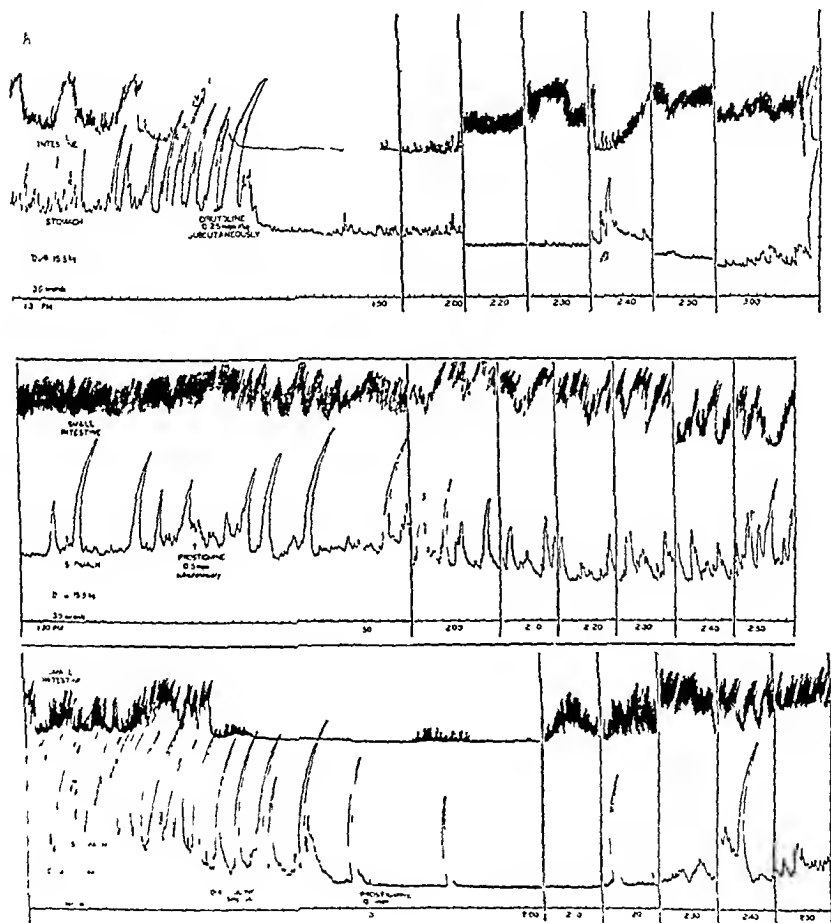


FIG 4

Dibutoline injected in a concentration of 0.25 mgm /kgm. subcutaneously (figure 5, A) relaxed the colon for a period of ninety minutes before a normal series of contractions occurred. This relaxation was of the same duration as that caused by atropine sulfate in the same concentration (figure 5, B). However, as these tracings show, dibutoline relaxed the small intestine for only seventy minutes,

the stimulating action of mecholyl, the concentration used was not great enough to block the stimulating action of barium chloride in a concentration of 1:25,000 (figure 2, C); however, a further addition of dibutoline, 1:50,000, was sufficient to overcome partially the effect of the barium chloride.

Barium chloride in a concentration of 1:25,000 caused a marked stimulation of the gut strips (figure 3, A) which required concentrations of dibutoline as high as 1:5,000 (figures 3, A, B) to bring the contractions of the strips back to normal, and even this concentration was not sufficient to prevent further stimulation by barium chloride. For comparative purposes the effect of atropine in 1:2,500 concentration, added after stimulation of the intestinal strips by barium chloride is shown (figure 3, D). In this case the contractions returned almost to normal for a time, then became very irregular. Although the simultaneous administration of 1:5,000 dibutoline and 1:25,000 barium chloride (figure 3, C) resulted, after a brief depression, in the typical barium chloride action, the addition of the same amount of dibutoline a few seconds prior to the addition of the barium chloride caused an almost complete inhibition of the barium chloride stimulation (figure 3, E). Atropine in a concentration of 1:8,000 was capable of inhibiting for a time the stimulation due to barium chloride (figure 3, F), then the strip (as in III, D) went into a spasm and all contractions stopped. Histamine, another musculo-stimulant drug like barium chloride, was shown in a single experiment to exert its stimulating action regardless of the presence of 1:10,000 dibutoline (figure 3, G).

Uterine: In a similar manner the effect of dibutoline was tested on isolated strips of uteri from four guinea pigs. No effects were noted when dibutoline was added in concentrations up to 1:20,000, but this dose and greater doses elicited increased contraction roughly equivalent to that caused by 1:100,000 histamine. This observation cannot be explained in the light of present pharmacological knowledge.

ANTISPASMODIC ACTION ON STOMACH, SMALL INTESTINE, AND COLON IN UNANESTHETIZED DOGS. Three dogs were prepared with gastrostomies and Thiry-Vella loops and the movements of the stomach and small intestine were recorded in the unanesthetized dogs on twenty different days in the manner of Plant and Miller (5). Dibutoline given subcutaneously in a concentration of 0.25 mgm./kgm. was effective in relaxing both small intestine and stomach (figure 4, A). This same concentration of the drug was also effective in preventing the increases in tonus and height of contractions resulting from the subcutaneous injection of 0.5 mgm. of prostigmine (figures 4, B, C). Dibutoline was equally effective with atropine in blocking the action of prostigmine given simultaneously with the antispasmodic drug and in overcoming the stimulation of previously administered prostigmine, both with the concentration used above and with 0.1 mgm./kgm. Oral doses of 10 mgm./kgm. of dibutoline to two dogs failed to have an effect on the motility of either stomach or gut, and a dose of 5 mgm./kgm. placed directly in the loop of small intestine also failed to elicit any response. However, oral doses of 50 mgm./kgm. were effective in decreasing the tonus and height of contractions.

An experiment was conducted to compare the ability of dibutoline with that of atropine in overcoming the depressant effect of acetylcholine on the blood pressure of an anesthetized dog. Drugs were injected in the femoral vein. Acetylcholine given in a concentration of 0.01 mgm./kgm. caused a 30 mm. (Hg) drop in the blood pressure. Atropine given in the same concentration was sufficient to prevent this decrease in blood pressure from a subsequent injection of acetylcholine, but 0.1 mgm./kgm. of dibutoline was required to prevent this blood pressure drop (figure 6).

ACTION ON THE BLADDERS OF UNANESTHETIZED DOGS. The technique of Langworthy, et al. (6) was used to obtain a kymographic record of the effect of dibutoline on the bladders of unanesthetized dogs, and to measure the ability of dibutoline to overcome the effect of parasympathetic stimulation, which, according to the accepted theory, results in an increased tone of the bladder mus-

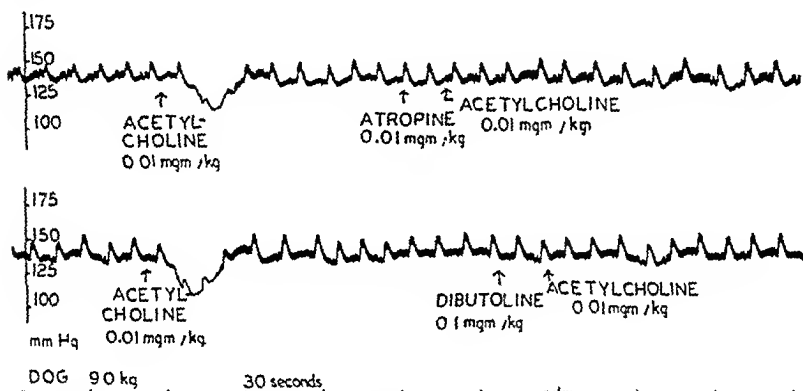


FIG 6

culature and a decreased tone of its sphincters, thus reducing the capacity of the bladder.

Three normal dogs were used. They were trained to lie under light restraint while normal saline at room temperature and under a constant head of pressure was allowed to flow into the bladder through a metal catheter until fluid was expressed around the catheter by bladder contraction, then inflow and kymograph were stopped and the bladder permitted to empty.

Measurements of the tonicity and capacity of the bladder and the urethral resistance to the outflow of urine were made according to the procedure of Winter (7). A tonicity reading in terms of "degrees of slope" was obtained by measuring the acuteness of the angle made by the rising line which recorded intravesical pressure on the kymograph drum. Likewise according to Winter (7) an estimate was obtained of the urethral resistance by determining the pressure at which fluid first appeared around the catheter after having traversed the entire urethra; the volume of saline needed to fill the bladder to the point where the urethral resistance was overcome was recorded as the capacity of the bladder.

while the period of relaxation following atropine was three times as long. After dibutoline there were no signs of restlessness, dilatation of the pupils, or dryness of the mouth as observed after atropine.

BLOOD PRESSURE AND PULSE EFFECTS. Subcutaneous injections of 0.25 mgm./kgm. of dibutoline in four dogs caused in each case a slight drop in pulse count in

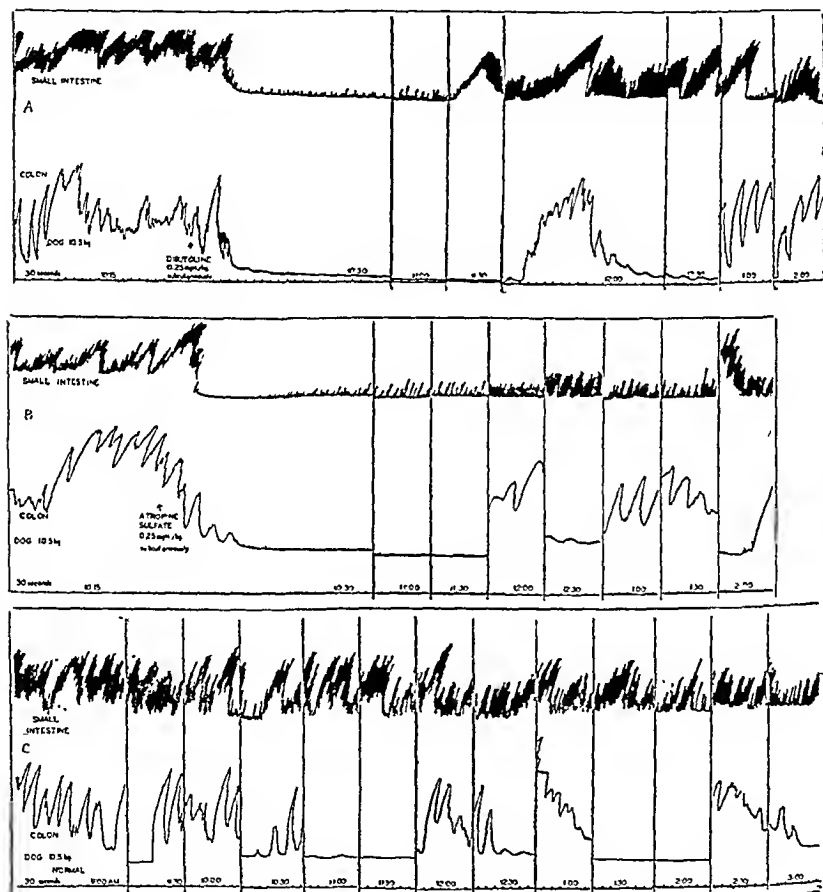


FIG. 5

the first five minutes following injection, followed by an increase of sixteen to thirty beats per minute in from ten to eighteen minutes, with a return to normal in thirty minutes. There was no noticeable effect on blood pressure from the same dose given intravenously to two anesthetized dogs; however, 2 mgm./kgm. produced a slight decrease.

the efficiency of a 0.25 mgm./kgm. dose of atropine sulfate in overcoming the action of mecholyl are also shown for comparison.

EFFECT ON SALIVARY SECRETIONS. In an effort to get some further insight into the mode of action of dibutoline, the effect of the drug on the secretions of the salivary glands, which respond entirely to nervous control was compared with the well known effect of atropine on the same process. Two dogs were anesthetized with pentobarbital sodium (30 mgm./kgm. intravenously). In each the chorda tympani and cervical sympathetic nerves were exposed and prepared for stimulation. Wharton's duct was exposed and a cannula attached to a drop recorder was inserted. Drugs were injected into the femoral vein. The results obtained are shown in table 1. Stimulation of the peripheral ends of the severed chorda tympani and cervical sympathetics brought the typical response and served to form a basis for comparing the actions of the drugs used later. Pros-

TABLE 1
Action of dibutoline and atropine on salivary secretions

	DIBUTOLINE EXPERIMENT DOG 1 (19.5 kgm.)	ATROPINE EXPERIMENT DOG 2 (16 kgm.)
	<i>drops</i>	<i>drops</i>
1. Stimulation of chorda tympani (weak stimulus for 5 seconds)	14	12
2. Stimulation of cervical sympathetic (strong stimulus for 15 seconds)	5	4
3. Prostigmine 0.25 mgm.	0	0
4. Acetylcholine 0.5 mgm.	15	20
5. Dibutoline 0.5 mgm./kgm.	0	
6. Atropine 0.5 mgm./kgm.		0
7. Acetylcholine 0.5 mgm.	0	0
8. Stimulation of chorda tympani (weak stimulus, 5 seconds)	0	0
9. Stimulation of cervical sympathetic (strong stimulus, 15 seconds)	4	3

tigmine had no effect on the secretions, but did prevent the destruction of a subsequent dose of acetylcholine. Dibutoline was as effective as atropine in blocking the action of acetylcholine and the electrical stimulation of the chorda tympani. The observation that stimulation of the cervical sympathetic after dibutoline caused secretion from the salivaries suggests that, like atropine, dibutoline acts to block only the cholinergic system.

BRONCHODILATING EFFECT. The lungs of three guinea pigs were perfused with non-oxygenated Locke-Ringer solution essentially according to the technique described by Sollmann and von Oettingen (8). The rate of flow of perfusion fluid was measured with a drop recorder. Histamine in such small quantity as 0.002 mgm. injected into the cannula caused a marked constriction of the bronchioles which could not be overcome effectively with additions of dibutoline in concentrations as high as 5 mgm. However, dibutoline was effective in preventing the normal bronchoconstricting action of pilocarpine.

In figure 7, A the average urinary bladder responses of three dogs are plotted. It can be seen that dibutoline given in a concentration of 0.5 mgm./kgm. subcutaneously caused no changes in any of the functions which exceeded the limits of normal variation. In the lower graph (figure 7, B) averages for two dogs are

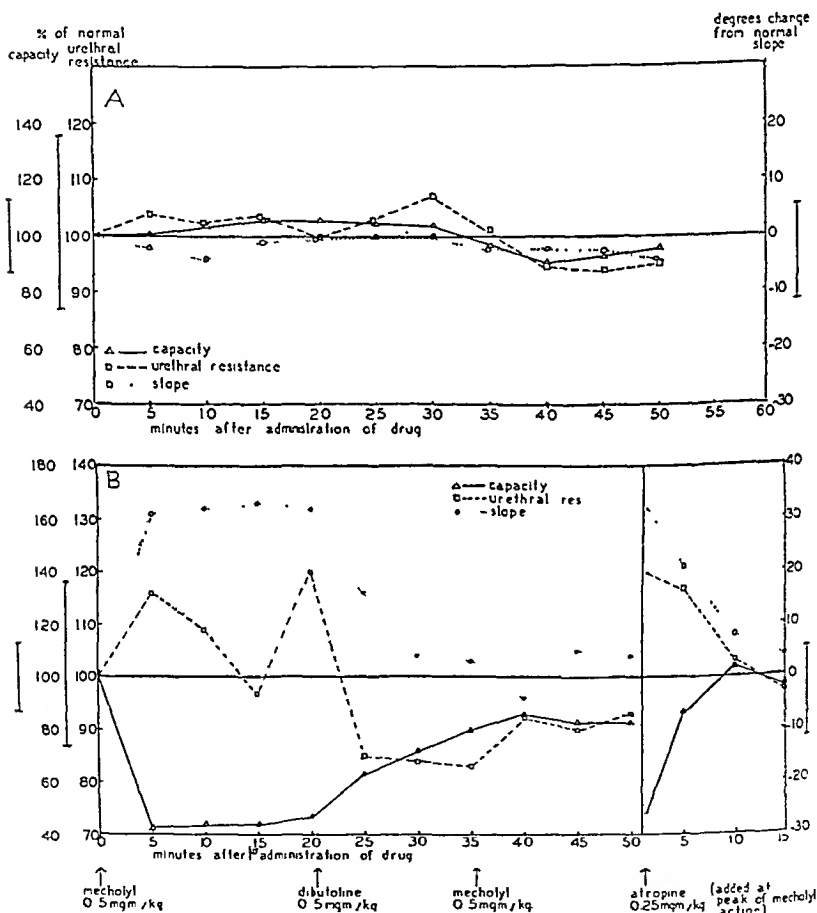


FIG. 7. Urinary bladder response to 0.5 mgm./kg. dibutoline (3 Dogs); side bars indicate limits of normal variation. Urinary bladder response to mecholyl before and after dibutoline. All drugs given subcutaneously.

recorded. Mecholyl caused a marked increase in slope (greater tonicity) and a marked reduction in the capacities of the bladders. Urethral resistance varied, but did not exceed the normal limits to an appreciable extent. Dibutoline given twenty minutes after the mecholyl not only overcame the effects of mecholyl, but also prevented the action of a subsequent dose of mecholyl. Data obtained on

EFFECT OF ROUTE OF ADMINISTRATION ON DETOXICATION OF SELENIUM BY ARSENIC^{1, 2, 3}

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The detoxication of selenium by arsenic was first reported in 1938 (1), when it was found that small amounts of arsenic (sodium arsenite) in the drinking water protected rats against the toxic action of selenium in seleniferous wheat which was included in the diet. Later work has shown that arsenic is effective against sodium selenite as well as the selenium which occurs naturally in wheat grown on seleniferous soils (2). With oral administration of both selenium and arsenic, detoxication has been satisfactory with hogs (3), dogs (4), poultry (5) and cattle (6), as well as with rats.

The mechanism by which arsenic counteracts the toxicity of selenium has not been explained but it has been suggested (7) that the arsenic combines with selenium in the gastro-intestinal tract and decreases the absorption of selenium. Research on the metabolism of selenium (8) would, however, indicate that arsenic does not interfere with absorption of selenium from the gastrointestinal tract. If the arsenic acts by preventing the absorption of selenium, it should be relatively ineffective as a detoxicant when given by subcutaneous injection. Likewise, arsenic given orally should not detoxify selenium given by subcutaneous injection.

EXPERIMENTAL. Nine groups of rats were used in the first series (series 15) as shown in table 1. Five female rats were used for each group. They weighed, from 50 to 60 grams when started on experiment. The average initial weight of rats in each group was 55.2 grams.

The diet was of the following composition:

	%
Wheat	82.5
Cascia	10.0
Salt mixture (Phillips-Hart No. 3)	1.0
Cod Liver Oil	1.5
Yeast	1.0
Lard	3.0

For groups 4, 5, and 6, sufficient sodium selenite was added to the diet so as to give a selenium content of 16.5 p.p.m. The drinking water for groups 2, 5, and

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DISCUSSION. In view of the relative concentrations used in overcoming the stimulating effects of mecholyl and barium chloride and the similarity of the action of dibutoline and atropine on the activity of isolated muscle, stomach, small intestine, colon, blood pressure, pulse effects, bladder musculature and sphincters, and the salivary secretions, it appears that the primary action of dibutoline, like atropine, lies in blocking the response to cholinergic stimulation. Dibutoline apparently blocks the muscarinic responses to acetylcholine and the parasympathomimetic drugs, whether they are excitatory as in the intestine and bladder or inhibitory as in the heart. However, the data presented here do not offer conclusive evidence that dibutoline is lacking in direct muscular effects. The drug seems deserving of clinical trial as an antispasmodic, especially since there are apparently no unfavorable side actions following its administration.

SUMMARY

Dibutoline has been shown to have favorable antispasmodic effects on smooth muscle when given by injection, but to be effective in oral doses, over 30 mgm./kgm. must be administered. It has been shown to be more effective in overcoming or blocking the stimulation or inhibition of smooth muscle due to neurotropic drugs, like acetylcholine, than in overcoming or blocking the action of musculo-stimulating drugs, like barium chloride. The mode of action of dibutoline appears to be similar to that of atropine.

The authors gratefully acknowledge the assistance in preparing the animals, and the suggestions and criticisms given by Dr. E. G. Gross during the progress of this work.

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8 contained 7.5 p.p.m. of arsenic (sodium arsenite). The solutions of selenium (sodium selenite) and arsenic (sodium arsenite) used for injections were made up in physiological saline solution. The consumption of seleniferous diet by groups 4, 5, and 6, and of arsenic-containing water by groups 2, 5, and 8, was measured and recorded daily. These values were used in computing the amounts of selenium and/or arsenic to be injected into the animals in groups 3, 6, 7, 8, and 9. All rats which died during the course of the experiment were autopsied and their livers examined for gross lesions. All rats surviving at the end of the experiment (48 days) were sacrificed and examined for gross liver lesions.

Two more series of rats were used to determine the ratio of arsenic to selenium for detoxication when both elements are given by subcutaneous injection. The rats in these two series were maintained on a stock colony ration. They were given selenium by subcutaneous injection as shown in table 2.

RESULTS AND DISCUSSION. The mortality of rats in the various groups (series 15) is shown in table 3. Deaths occurred in only four of the groups: 4, 5, 7,

TABLE 2
Series 16c and 16d: average life of rats

SERIES NO.	GROUP NO (5 RATS TO A GROUP)	DAILY DOSE IN MCM /KG*	
		Selenium	Arsenic
16c	1 (8 days)	1 5	1 5
16c	2 (all lived)		1 5
16c	3 (4 days)	1 5	
16d	1 (1 died 6 days, 4 lived 65 days)	1 0	1 5
16d	2 (all lived)		1 5
16d	3 (4 died 18.5 days, 1 lived—severe cirrhosis)	1 0	

* Subcutaneous injection.

and 8. It will be noted that regardless of the route of administration of either of the elements the arsenic was effective as a detoxicant. The mortality of groups 5 and 8, which received arsenic, was not as high as in groups 4 and 7 where no arsenic was given. The rats in group 7 which received selenium by subcutaneous injection in amounts equal to the oral selenium consumption in group 4 all died within seven days after they were put on experiment. This would indicate that the sodium selenite which was consumed in the diet was not as toxic as when given by subcutaneous injection. It has been shown that the orally ingested selenium is not completely absorbed from the gastro-intestinal tract (8). Furthermore, the selenium which is absorbed from the gastro-intestinal tract passes through the portal vein into the liver before it enters the systemic circulatory system which would allow for hepatic detoxication and thus protect the body tissues from some of the selenium which is absorbed from the gastro-intestinal tract. On the other hand, selenium which is injected into the subcutaneous tissues is absorbed into the systemic circulatory system and has an opportunity to contact many tissues before it is subjected to detoxication by

TABLE 1
Series 15: treatment of rats

GROUP NO.	TREATMENT	
	Selenium (sodium selenite)	Arsenic (sodium arsenite)
1	None	None
2	None	Orally, 7.5 p.p.m. in drinking water
3	None	Subcutaneously, amount equal to average intake of rats in Group 2 on previous day
4	Orally, 16.5 p.p.m. in diet	None
5	Orally, 16.5 p.p.m. in diet	Orally, 7.5 p.p.m. in drinking water
6	Orally, 16.5 p.p.m. in diet	Subcutaneously, amount equal to average intake of rats in Group 5 on previous day
7	Subcutaneously, amount equal to average intake of rats in Group 4 on previous day	None
8	Subcutaneously, amount equal to average intake of rats in Group 5 on previous day	Orally, 7.5 p.p.m. in drinking water
9	Subcutaneously, amount equal to average intake of rats in Group 6 on previous day	Subcutaneously, amount equal to average intake of rats in Group 8 on previous day

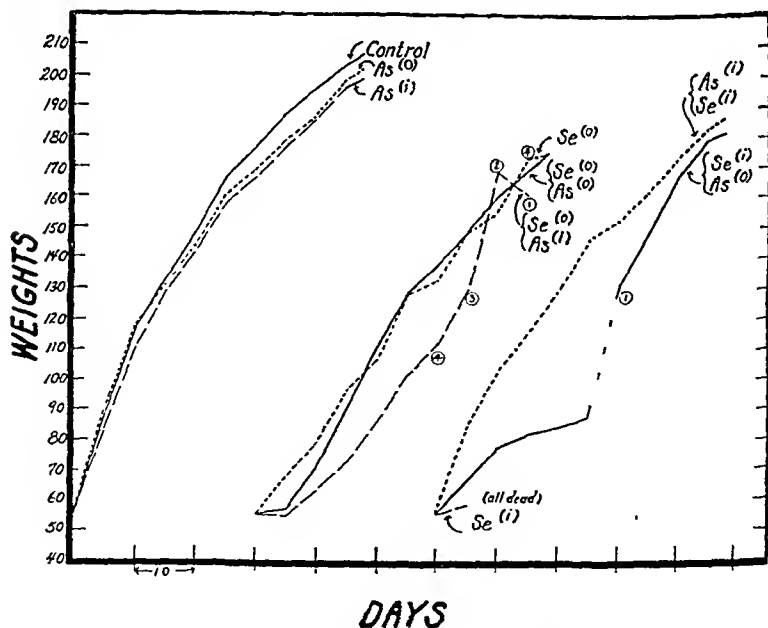


FIG. 1. GROWTH CURVES OF RATS IN SERIES 15

Weights in grams

Figures in circles—rats remaining alive

(o)—element administered orally

(i)—element administered by subcutaneous injection

substantiate the theory that the arsenic acts by inhibiting the absorption of selenium from the gastro-intestinal tract.

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the liver. The rats in group 7 (table 4) all died before liver lesions developed and they had received an average of only 0.268 mgm. of selenium. The rats which died in group 4 had developed moderate to severe liver cirrhosis and had received an average of 3.08 mgm. of selenium at the time of death.

In the two other series (16c and 16d) it was shown further that arsenic administered subcutaneously would counteract selenium given by the same route. It was also shown that the amount of selenium which 1.5 mgm./kg. of arsenic

TABLE 3
Series 15: mortality of rats in experiment

GROUP NO.	TREATMENT	% MORTALITY DURING EXPERI- MENT (48 DAYS)	AVERAGE NO. OF DAYS ON EXPT. OF RATS WHICH DIED DURING EXPERIMENT
1	No Se., No As.	0	
2	No Se., As. (Orally)	0	
3	No Se., As. (Subcut.)	0	
4	Se. (Orally), No As.	80%	30.2
5	Se. (Orally), As. (Orally)	20%	40.0
6	Se. (Orally), As. (Subcut.)	0	
7	Se. (Subcut.), No As.	100%	6.2
8	Se. (Subcut.), As. (Orally)	80%	9.5
9	Se. (Subcut.), As. (Subcut.)	0	

TABLE 4
Series 15: condition of livers from rats

GROUP NO.	CONDITION OF LIVERS OF RATS WHICH DIED DURING EXPERIMENT	CONDITION OF LIVERS OF RATS WHICH SURVIVED
4	Moderate to severe cirrhosis	Mild cirrhosis, slight atrophy
5	One, severe "hobnailed" cirrhosis	Mild to severe cirrhosis. One, severe cirrhosis with atrophy
6		Slight necrosis with atrophy
7	All died before lesions developed	
8	Three died first week. One died after 25 days, some necrosis	One survived, no lesions
9		No lesions

will detoxicate is between 1.0 and 1.5 mgm./kg. when both elements are administered subcutaneously.

The growth of the rats as shown in Figure 1 is of particular interest. Arsenic alone reduced the growth rate only slightly, while in the presence of selenium the arsenic greatly improved the growth of the animals.

SUMMARY

It has been demonstrated that the detoxication action is independent of the route of administration of either the selenium or the arsenic. This would not

Local infection of mice. A method identical with that described by Morgenroth and Abraham (7) for local infection with *Streptococcus hemolyticus* was used. Doses of 0.1 ml. of undiluted cultures of *C. diphtheriae* in tryptose-phosphate broth were injected subcutaneously into the abdominal wall of mice. As 0.1 ml. of a 10^{-2} culture dilution still produced subcutaneous abscesses and necrosis of the skin, the actual infective dose corresponded to about 100 minimal doses. All controls developed abscesses and necrosis. The infective organism was always recovered from the site of the lesion. Immediately after infection, different dilutions of penicillin dissolved in 1 ml. saline were injected subcutaneously into the site of infection. The mice were killed 48 hours after infection and treatment, and cultures were taken from the site of the lesion.

GENERALIZED INFECTION OF MICE. Overnight cultures of the bacillus in tryptose-phosphate broth, diluted to 10^{-2} , were injected into mice in 1 ml. 5% mucin. In more recent experiments, slant growths on blood agar suspended in broth were brought to a turbidity corresponding to that of the broth cultures (55-60% light transmission on the Fisher Electrophotometer, Filter A) and diluted to 10^{-2} . The infective dose corresponded in both cases to 100 average lethal doses.

Diphtheria infection of guinea pigs. One ml. of an overnight broth culture diluted to 1 to 10 in saline was injected subcutaneously into guinea pigs of 300-500 g. of weight. The number of cells injected in different experiments varied between 1,400,000 and 8,500,000.

3. EXPERIMENTAL. (a) *Bacteriostatic activity of penicillin in vitro.* The findings of Abraham, Florey, and associates (2) were confirmed in this laboratory.

The growth-inhibiting concentration of penicillin *in vitro*, as tested with the serial dilution method in papain digest glucose broth or in tryptose phosphate broth, corresponds to 1.5 to 1.25 units/ml. as measured by cup test against *S. aureus* #209. Partial growth inhibition was observed with concentrations of 0.35 units/ml. This is an approximately 100 times higher concentration than is required for the growth inhibition of β -hemolytic streptococci and of pneumococci.

Furthermore, it was studied whether penicillin exerts any influence on the toxin formation by *C. diphtheriae*. Intradermal injections of rabbits with 0.2 cc. of broth cultures after 20 hours' contact with penicillin at 37°C. showed that in all tubes in which the growth of the organism was not completely inhibited, diphtheria toxin was formed. The characteristic skin lesions produced by cultures in which the growth was partly inhibited were, however, smaller (15-22 mm. diameter) than those from fully grown cultures which contained less than 0.3 units/cc. penicillin or no penicillin at all (28-38 mm. diameter). This indicates that the toxin formation is not influenced directly by penicillin; the amount of toxin formed was decreased to the extent to which bacterial growth was inhibited.

(b) *Local effect of penicillin on C. diphtheriae lesions in mice.* Penicillin doses as high as 800 to 2000 units failed to influence the development of lesions. Cultures from most of these lesions showed growth of *C. diphtheriae*. In only 4 out of 25 cases no growth was obtained.

(c) *Systemic activity of penicillin on diphtheria bacillus infection of mice.* (Appendix: Sulfadiazine). The systemic activity of penicillin on lethal infections with *C. diphtheriae* was tested in mice infected intra-abdominally with suspensions of *C. diphtheriae* in 5% mucin.

Infection. According to the data on the virulence of *C. diphtheriae* cultures, given in table 1, it appears that the LD₅₀ corresponds to 1 ml. of the 10^{-5} dilution

THE ANTIBACTERIAL ACTIVITY OF PENICILLIN IN EXPERIMENTAL INFECTIONS OF MICE WITH *C. DIPHTHERIAE*

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1. INTRODUCTION. The diphtheria bacillus as a gram-positive organism was found to be fairly sensitive *in vitro* to the bacteriostatic activity of penicillin (Chain and Florey (1); Abraham and associates (2); Fleming (3)). The activity of penicillin on experimental infections with *Corynebacterium diphtheriae* *in vivo* has not yet been studied so far; this might be due to the difficulty of producing experimental infections without interference with the lethal action of diphtheria toxin.¹ A bacterial infection without toxemia has been obtained recently by Seligmann and Jungeblut (5) by injecting very large doses of diphtheria bacilli into rats. It could be assumed that the white mouse would respond similarly to an infection with *C. diphtheriae*.

The mouse is considered highly resistant to infection with *C. diphtheriae*, and several investigators (Kolle and Schlossberger; Hippke; Ulrich; Wolf²) reported more or less unsatisfactory results. As to the toxin effect, a recent work of Petherick (6) shows that mice are 170 times more resistant than guinea-pigs to diphtheria toxin given intravenously, while the skin of the mouse is 20,000 times more resistant. However, it was found by us that intra-abdominal infection of mice with *C. diphtheriae* in mucin is successful and offers the first reliable method by which to study chemotherapeutic activity. In this type of experiments, the influence of the toxin produced by the micro-organism plays only an insignificant rôle.

This method of infection enabled us to investigate the *antibacterial* action of penicillin against *C. diphtheriae*. Experiments on the antitoxic activity *in vitro* and *in vivo* were included in these studies.

2. MATERIAL AND METHODS. *Corynebacterium diphtheriae* No. 9060, Gravis Type, obtained from the American Type Culture Collection, was used throughout this study. Overnight cultures in tryptose-phosphate broth (Difco) or papain digest glucose broth were used for *in vitro* experiments. In animal experiments, broth cultures on tryptose-phosphate broth and slant growths on blood-agar, suspended in broth, were injected in appropriate dilutions. For organ cultures, solid media containing potassium tellurite were used.

The diphtheria toxin No. 3915, utilized for neutralization experiments, was kindly furnished us by Dr. A. J. Weil of Lederle Laboratories.

A commercial antiserum (Lederle, No. 54H4892), containing approximately 4000 units/ml., was used as antitoxin.

¹ According to Neter and Will (4), penicillin does not neutralize tetanus toxin *in vitro*. Their findings have been confirmed in this laboratory as far as lower concentrations of penicillin are concerned. Higher concentrations (e.g., 2500 u./cc.) and prolonged contact at 37°C. neutralize 2 MLD of tetanus toxin.

² Quoted from Seligmann and Jungeblut (4).

theria bacillus. As was to be expected, lower doses administered repeatedly; i.e., 10-25 units to a total of 40-100 units/20 g. penicillin, gave similar therapeutic results.

Mice surviving infection following chemotherapeutic treatment were completely sterilized as shown by cultures made from the organs.

Appendix. We used the same method for testing the antibacterial activity of sulfonamides in diphtheria infection.

Table 3 shows that a single oral treatment with 50 mg./kg. sulfadiazine protected 50% of the mice against infection with 100 MLD. With higher doses or repeated treatment, all animals could be protected.

It might be mentioned in this connection that antitoxic serum injected subcutaneously in doses as high as 40 units/20 g. mouse was almost ineffective in this type of infection. This is in agreement with the observations made on rats by Seligmann and Jungeblut (5). The inactivity of antitoxin treatment in mucin-infected mice can be considered as an additional proof that the infection is mainly bacteriemic and that the deaths of the animals is not due to toxin formation.

TABLE 3
Activity of sulfadiazine against C. diphtheriae infection of mice

TOTAL ORAL DOSE	NUMBER OF TREATMENTS	NO MICE	NO SURVIVORS
<i>g kg mouse</i>			
0.012-0.025	1	10	0
0.050	1	20	12
0.125	1	5	5
0.250	1	5	5
0.5-1.0	1	10	8
2.0-4.0	4	10	10

Immunity. Mice surviving infection by treatment with penicillin and sulfadiazine were found resistant to a re-infection with 100 MLD of culture injected in mucin intra-abdominally after 20 days. The mice therefore developed an immunity which could be demonstrated a long time after penicillin was eliminated from the body. The diphtheria bacilli destroyed by the activity of penicillin apparently formed the antigen responsible for the immunity.

The entire group of these experiments seems to indicate that the intra-abdominal infection of mice with *C. diphtheriae* produced a diphtherial infection of bacteriemic character which could be controlled by treatment with penicillin or sulfonamides, e.g., sulfadiazine. The activity of these agents appeared to be a genuine chemotherapeutic effect: the survival of the treated animals after proper doses of penicillin or sulfadiazine was due to the disappearance of the causative organisms from the site of the infection and the circulation.

No information as to the activity of penicillin on the diphtheria toxin could be derived from these observations. It seemed appropriate to investigate this question in order to acquire more complete knowledge on the value of penicillin under conditions more similar to the pathology of diphtheria infection in human beings.

of the broth culture, or of a slant suspension of equal turbidity. Variations in the lethal effect of the diluted cultures were found to depend on the number of bacterial cells. These could not be kept absolutely constant in all experiments. The virulence of slant suspensions seemed to be more constant than that of broth cultures.

Most untreated control animals died within 48 hours. Peritoneal smears stained with methylene blue showed numerous intra- and extra-cellular bacilli

TABLE 1

Mouse virulence of C. diphtheriae injected intra-abdominally with 1 cc mucin

DILUTION OF THE CULTURE*	NUMBER OF MICE	SURVIVORS
		per cent
10 ⁻²	21	23.8
10 ⁻³	181	16.6
10 ⁻⁴	85	30.6
10 ⁻⁵ †	52	52
10 ⁻⁶	33	76

* Or of a slant suspension of same turbidity.

† Corresponding to 140-900 cells as determined by plate-count

TABLE 2

Activity of penicillin against C. diphtheriae infection of mice infection

Intra-abdominal injection of broth or slant suspension diluted 10⁻³ in 1 cc mucin

SINGLE DOSE PENICILLIN PER 20 G SUBCUTANEOUS	SINGLE TREATMENT NUMBER OF		TREATMENT REPEATED 4 TIMES NUMBER OF	
	Mice	Survivors	Mice	Survivors
		per cent		per cent
Controls 10 ⁻³	106	6.6		
Controls 10 ⁻⁴	32	6.2		
5-10 units	50	30	5	80
25 units	45	28.8	5	100
50 units	35	28.5	5	60
100 units	25	68	5	60
200-250 units	20	80	5	100
500-1000 units	10	100	5	80

Heart cultures on solid agar media gave growth of the infective organism. In the majority of mice, there were no pathological findings, which indicated that the infection was purely bacteriemic. Only in a few cases, the adrenals appeared hemorrhagic, suggesting that toxemia might have developed.

Therapeutic experiments. Penicillin was administered subcutaneously 10-20 minutes before infection. For repeated treatment, 3 successive injections were given at 2-hour intervals. The therapeutic results are given in table 2.

The table shows that single doses of 100 units/20 g. or more protect the majority of mice infected with 100 times the minimum lethal dose of diph-

Generally, no growth was obtained by culturing from a local lesion; only occasionally a few colonies could be found. No bacilli could be recovered from any of the organs. The animals died by the effect of diphtheria toxin.

Therapeutic experiments. Penicillin was given subcutaneously 20 minutes before infection; treatment was repeated 3 to 4 times at 2-hour intervals during the first three days. High doses, such as a total of 50,000 to 110,000 units/kg., caused only a delay of death up to 4 to 7 days (table 5). More than 3 weeks' survival was observed in only an insignificant number of animals. Infection of

TABLE 4
Action of penicillin added to diphtheria toxin in vitro
0.2 cc mixture incubated for 5½ hours, injected intradermally

TOXIN DILUTION	RABBIT A		RABBIT B		RABBIT C	
	Control	Penicillin 2500 u/cc	Control	Penicillin 5000 u/cc	Control	Penicillin 300 u/cc
1:1000	37 mm *	34 mm.	37 mm	29 mm	36 mm	0
1:2000	23 mm	18 mm	20 mm	trace	18 mm.	0
1:4000	trace	trace	trace	0	20 mm.	0
1:8000	0	0	0	0	trace	0

* Diameter of the lesion.

TABLE 5
Activity of penicillin in the diphtheria infection of guinea pigs with live bacilli

INFECTION (5 C)	PENICILLIN SINGLE DOSE	REPEATED TIMES	NO GUINEA PIG	NUMBER ALIVE AFTER DAYS									
				1	2	3	4	5	6	7	8	9	Survived
1 cc. 10 ⁻¹ susp	u/kg		17	16	1	0							
	Controls		3	3	2	1							
	500 u/kg	5	3	3	3	3							
	2000 u/kg	8	3	3	3	2							
	2500 u/kg	11	3	3	3	2							
	5000 u/kg	11-15	8	8	6	5							
	10,000 u/kg	11	3	3	3	3							
	20,000 u/kg	12	3	3	3	3							

guinea pigs with lower doses, e.g., 10⁻² suspensions, failed to show better results of the penicillin treatment. Thus, in the bacterial infection of guinea pigs which developed a fatal toxemia, penicillin had only negligible activity.

(f) *Activity of penicillin on diphtheria toxin in guinea pigs.* Guinea pigs were injected subcutaneously with 1 ml/kg of a mixture of 5 LD diphtheria toxin and 3915 (1:50) incompletely neutralized with antitoxin 1:20,000 to 30,000. Nine out of 11 guinea pigs treated subcutaneously with total doses of 45,000, 170,000, and 300,000 units/kg. penicillin died. Death occurred at the same time as in 10 out of 18 untreated controls. Thus, even these extremely high doses of penicillin did not influence in the least the toxic action of diphtheria toxin.

(d) *Activity of penicillin on the action of diphtheria toxin in vitro.* Twenty-one rabbits were injected intradermally with mixtures of penicillin and diphtheria toxin of various concentrations incubated at 37°C. for different intervals. Each rabbit was injected on one side with a series of incubated toxin-saline dilutions alone, on the other with the corresponding penicillin-toxin mixtures. It was found that doses as high as 5000 units/cc. penicillin had only a slight effect on diluted diphtheria toxin corresponding to 1-4 necrotizing doses. Higher concentrations of penicillin, such as 10,000 u./cc., and after 5 hours' incubation, gave

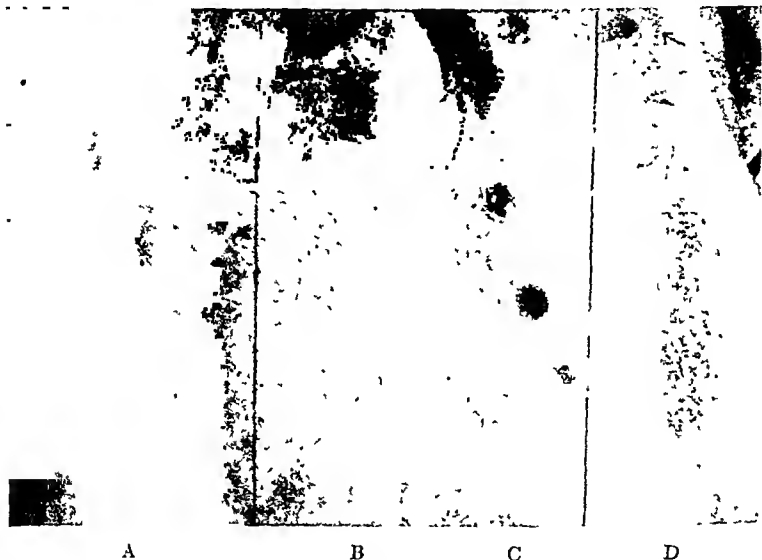


FIG. 1

- A & C Rabbit injected intradermally with toxin saline mixtures 1:1000, 1:2000, 1:4000, 1:8000
 B Injected with the same dilutions of toxin + crystalline penicillin solution 10,000 u/cc
 D Injected with the same dilutions of toxin + commercial penicillin solution 10,000 u/cc Note that the highest toxin concentration (1:1000) has not been completely inhibited (↑) in this experiment.

complete inhibition of the toxin effect as tested on the skin of rabbit. It should be mentioned that crystalline penicillin G (potency 1660 units/mg.) gave the same effect as commercial samples (fig. 1). This seems to indicate that the toxin inhibition depends on the penicillin component. A typical experiment made with commercial penicillin is reported in table 4.

(c) *Activity of penicillin on C. diphtheriae infection in guinea pigs.* Infection. Untreated animals injected subcutaneously with bacterial suspensions died within 30 to 56 hours. Necropsy revealed hemorrhages and necrosis at the site of injection, hemorrhages in the adrenals, pleural and sometimes pericardial exudates.

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4. CONCLUSIONS. In the experiments presented in the foregoing paragraphs, we disrupted the natural entity of the pathology of an infection with *C. diphtheriae*. By this way, we could study separately the effect of chemotherapy on a bacteriemic infection and on the natural toxemic condition. It was found that penicillin as well as sulfadiazine exert a striking antibacterial effect on the bacteriemic infection produced in mice by intra-abdominal infection with suspension of *C. diphtheriae* in mucin; anti-serum was inactive in this infection. Penicillin, on the other hand, was inactive in the toxemic infection of guinea pigs which, as to its pathology and antitoxin sensitivity, is more comparable to the human infection. Guinea pigs injected with lethal doses of toxin-antitoxin mixtures did not react at all to penicillin treatment, while the death of animals injected with bacterial suspensions could be somewhat delayed. We consider that the latter effect depends on an antibacterial mechanism and not on an antitoxic action; penicillin delays partially the bacterial development and, consequently, the toxin effect.

These results suggest that under the practical conditions of diphtheria in human beings, the preponderance of toxemia will require antitoxic treatment with anti-serum. However, there might be occasions, e.g., prophylaxis of exposed contacts, very early cases and carriers, where the anti-bacterial treatment with penicillin might be helpful.³ From our experiments, it furthermore might be seen that in such cases, systemic treatment with penicillin will most likely be more active than topical administration.

SUMMARY

1. Experimental systemic and local infections of mice with *C. diphtheriae* are described. In the systemic, principally bacillary, infection, penicillin exerted a marked activity, while it had no effect when administered topically on the local infection.

2. Penicillin had no effect on diphtheria toxin in guinea pigs injected with lethal doses of toxin-antitoxin mixtures.

3. High doses of penicillin delayed the death of guinea pigs infected with cultures of *C. diphtheriae*. The delay of death is considered due to a decrease of toxin formation induced by the antibacterial effect of penicillin.

4. Cultures of diphtheria bacilli grown in presence of penicillin do not lose their toxigenic properties if tested on the skin of rabbits.

5. Only extremely high doses of penicillin (10,000 units/cc.) neutralize diphtheria toxin *in vitro*.

6. It is concluded that penicillin will not be effective in human cases characterized by toxemia, while it might be useful in certain conditions of predominantly bacillary character, such as very early cases or carriers.

Acknowledgment. The authors are indebted to Dr. R. J. Schnitzer for numerous suggestions received during the course of this investigation.

³ In a recent pamphlet issued by the War Production Board, April 1, 1945, "The Indications, Contra-Indications, Mode of Administration and Dosage for Penicillin," diphtheria is mentioned under the heading "Indications in Group II," which "will require additional experimental work."

weighings permitted one to detect this and thus avoid the error introduced by the drop in blood pressure which accompanies starvation.

We have also administered orally the active marine oils, described later, to three hypertensive dogs, with drops in blood pressure comparable to that observed in the rat. The blood pressure of these dogs was determined by puncture of the femoral artery with direct readings on a mercury manometer. It is impracticable to use the dog for systematic assays, but the results indicate that the observed effects are not limited to the rat.

MARINE OILS. We have investigated the common commercially available marine oils for their blood pressure reducing activity. This varied considerably in oils of various sources and sometimes in different samples of oils derived from the same species. Active preparations have been obtained from residues of cod, dogfish, hake, basking shark and skate liver oils. Sardine body oil was also found to be slightly active as was also crude fish oil obtained by distillation, principally of menhaden. One sample of a fresh water fish (Burbot liver) was found to be entirely inactive.³

The commercially available samples of cod liver oil were found to be entirely inactive as were also some samples of the concentrates obtained from this source. On the other hand, some samples of concentrate (containing 50,000 to 100,000 units of vitamin A) per gram were found to be active and first prompted the present investigation (1).

In table 1 are reproduced the effects of thirty-one fractions of oils derived from the four species of fish, designated in the first column. In the second column are recorded the distillation temperature at which the oils were derived when distilled at pressures between 6 to 11 micromillimeters.⁴ The vitamin A content of the various fractions is given in the third column; their blood pressure reducing properties in the last three columns. Each fraction was tested without further treatment (column 4), following oxidation with thirty percent hydrogen peroxide at room temperature in slightly alkaline solution (column 5), and following saponification with aqueous alcoholic potash, acidification and oxidation (column 6). Fractions which in doses of 5 grams per rat reduced the blood pressure 15 mms. or more, for a period of at least two days are designated in the table as active (A). Those in which the blood pressure at this dosage level was reduced between 10 and 15 mms. as slightly active (SA); between 5 and 10 mms. as minimally active (MA). Obviously, the last named must be considered as manifesting only questionable activity.

It is evident from table 1 that there is no correlation between the vitamin A content of the various oils investigated and their effectiveness in reducing the blood pressure. Moreover, fractional distillation, although effecting to some extent the separation of the active material is not a satisfactory procedure for concentrating this material which apparently is carried over at a considerable

³ Most of the oils used in the present study were supplied by Eli Lilly and Co. The others were furnished by Distillation Products, Inc., Parke, Davis and Co., The Winthrop Co., The Abbott Laboratories, The National Oil Products Co., Industrial Chemical Sales, Lamer Industries, Procter and Gamble and Bogalusa Tung Oil, Inc.

⁴ We are indebted to Mr. A. Lee Caldwell of The Lilly Research Laboratories for supplying these fractionated oils and the data on their vitamin content.

THE PREPARATION OF EXTRACTS FROM OXIDIZED MARINE AND OTHER OILS FOR REDUCING THE BLOOD PRESSURE IN EXPERIMENTAL AND HUMAN CHRONIC HYPERTENSION

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In earlier reports (1, 2) it has been shown that various oxidized marine oils, when administered orally to hypertensive rats, reduces their blood pressure in a way similar to that of renal extracts (3). In view of the expense and technical difficulty involved in preparing active extracts from kidney tissue, compared to the less laborious procedure of obtaining equally effective agents from readily available marine oils, the latter appeared to be a better source of the desired product. We have, therefore, investigated a number of marine, vegetable and animal oils, as sources of this material as well as methods for the preparation and concentration of extracts suitable for administration to animals or to human patients. Our results have consistently shown the effectiveness of these products in the experimental animal (rats and dogs) and show promise in preliminary studies on man. The present paper reports details of the source, preparation and methods of concentration of the effective agent.

METHODS OF ASSAY. The effectiveness of the various preparations described later was determined on rats rendered hypertensive by compression of the renal parenchyma (4). Oral administration of the extracts by incorporating them in the animal's food has been used exclusively. This avoids the effects of a non-specific depression of the blood pressure which may follow the parenteral administration of various noxious agents and which are responsible for some of the effects observed following the administration of certain renal extracts (5, 6).

Groups of six hypertensive rats were used in each assay. The animals had been operated upon at least three months previously. In this way the spontaneous fluctuations which are observed in the months immediately following operation are avoided. After a preliminary daily training period for several weeks, the blood pressure remains essentially constant except for a slowly progressive rise which continues until the death of the animal (7). The mean blood pressures of the animals ranged from 150 to 200 mm. with an average pressure of the group of six used in each assay varying from 165 to 175. In this way one compensates for the fact that the blood pressure reducing capacity of extracts varies with the blood pressure of the animal and renders the results of separate experiments comparable.

The blood pressures were determined at the same time of the day by the procedure previously described (8). Experiments were discarded in which, due to distastefulness of the oil-mixtures, the animals refused to eat and lost a significant amount of weight. Daily

¹ Aided by grants from the John and Mary Markle Foundation, The National Life Assurance Co., and Mr. Alfred Starr.

² This work was begun at the Bowman Gray School of Medicine, Winston-Salem, N. C. The clinical data presented are based on observations of patients at The North Carolina Baptist Hospital.

mum activity. It would thus appear that some of the precursor of the active principle is present in the oil as a stable ester, and that only the compound liberated on hydrolysis is susceptible to oxidation and gives rise to the active agent.

VEGETABLE OILS. The presence of the active oil in several marine oils and its apparent production by oxidation of the esterifiable fraction suggested a search in other animal and vegetable fats for a source of the active agent. We have investigated the following oils both in their usually available form, as well as after oxidation and after oxidation of the hydrolyzed material: Almond (sweet), cottonseed, hydrogenated corn, soya bean, peanut, sesame, tall, olive, linseed and grape seed. Oils of animal origin (commercial red oils) were also studied.

Of the above-mentioned oils only tung oil showed unquestionable activity. The other oils investigated manifested none, or at most minimal activity. Some samples of tung oil showed an activity comparable to the most potent fish oils which we have encountered. Since this oil is readily and cheaply available it may offer a source for obtaining the active material superior to that of marine oils. Unfortunately, the yield was not consistently good from all specimens of oil which were obtained at different times. Moreover, the tendency of tung oil to solidify and to precipitate β -eleostearin on exposure to air and sunlight, renders difficult the extraction of the active principle. The use of the oil directly, except in very small doses, is impossible because of its unsavory taste and toxicity.

In Figure 1 are reproduced typical assays on active preparations of tung oil. As seen in the uppermost curve of this figure, the activity was manifest in the unoxidized oil. It may be assumed that the active principle is either present as such in the fresh oil or was produced during the process of refining. Further oxidation of the oil, as in the case of marine oils (fig. 1), appears to increase the activity further but this is not striking and the increased yield, if any, does not compensate for the technical difficulties induced by the undesirable secondary changes occurring during oxidation.

Although tung oil when administered in the small doses used in these experiments is not obviously toxic to rats, it is conceivable that the observed lowering of the blood pressure is a result of intestinal irritation, or other toxic effect of the oil. The improbability of this conclusion is shown by the third curve of Figure 1, in which a comparable effect on the blood pressure was obtained by administering an aqueous extract derived from tung oil by the procedure described in a subsequent section.

CLINICAL STUDIES Because of the normally great fluctuations in the blood pressure of the hypertensive patient and the spontaneous drops which occur on prolonged bed rest, the evaluation of the effectiveness of therapeutic agents on the blood pressure is difficult and requires many months of hospitalization under carefully controlled and constant conditions. We have thus far studied only three patients adequately to determine the effects of administering active oxidized oil on the blood pressure. Two other patients were given an aqueous concentrate with results which will be reported elsewhere.

In figure 2 is reproduced the blood pressure curve of a twenty-one year old male patient suffering from hypertension secondary to chronic glomerulo-nephri-

range of temperatures. In general, however, the active material distills over at a temperature of 215° with a second active fraction appearing in the distillate at about 250°.

TABLE 1
The pressure reducing activity of various fractions of four marine oils

SOURCE OF OIL	DISTILLATION TEMPERATURE	VITAMIN A CONTENT	EFFECTIVENESS IN REDUCING THE BLOOD PRESSURE		
			Untreated	Oxidized	Hydrolyzed and oxidized
Dogfish oil	°C.	Units per gram			
	200	240,000	A	A	A
	225	44,000	I	MA	A
	230	4,000	I	SA	A
	235	900	I	I	MA
	240	200	I	I	MA
	250	100	I	I	SA
	255	100	MA	MA	A
Hake liver oil	Residue	100	I	I	I
	210	215,000	I	MA	A
	215	40,000	MA	MA	A
	220	6,750	I	MA	A
	225	2,300	I	SA	MA
	230	500	I	SA	MA
	235	100	I	I	I
	240	100	SA	MA	A
Basking shark liver oil	Residue	100	I	MA	A
	100	Trace	I	I	I
	120	Trace	I	SA	SA
	130	Trace	I	MA	MA
	150	2,200	I	I	MA
	230	1,200	I	I	I
	250	100	I	I	MA
	255	None	I	I	SA
Skate liver oil	Residue				I
	165	635	A	A	A
	215	3,900	SA	MA	A
	230	180	I	SA	MA
	242	None	I	SA	SA
	245	None	I	I	I
	248	None	I	I	I
	Residue	None	MA	MA	SA

A comparison of the last three columns in the table also shows that oxidation or hydrolysis prior to oxidation in many cases fails to impart or increase the activity of certain oil fractions. In some cases oxidation alone increases the activity. In others, prior saponification is necessary in order to obtain the maxi-

discharge of the patient from the hospital on October 1, 1944. Blood pressure readings were made by three different members of the nursing, interne and resident staff of the hospital.⁵ The six daily readings obtained in this way were averaged and the resulting readings are recorded in the chart. In this way the inevitable fluctuations are eliminated in the final value which is composed of at least forty-two separate readings.

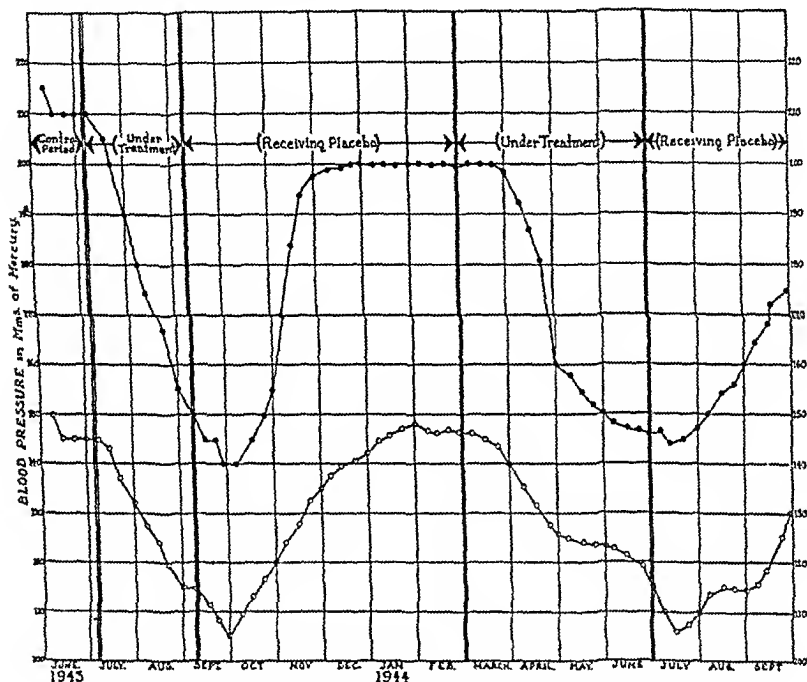


FIG. 2. THE BLOOD PRESSURE OF A HYPERTENSIVE PATIENT TREATED WITH OXIDIZED MARINE OIL

Each point on the curve is the average of at least forty-two readings obtained during the period of observation. The patient was alternately receiving an active oil (as tested on hypertensive rats) during the periods of July 7 to September 7, 1943, and February 28 to June 30, 1944, and an inactive oil as a placebo during the periods of September 7, 1943, to February 28, 1944, and July 1 to October 1, 1944.

- systolic blood pressures.
- diastolic blood pressures.

It is evident from the curve that, as in the experimental animal, the administration of an active oil to this patient resulted in a definite decline of the blood pressure. It should be noted that there is a latent period of several weeks following the beginning of therapy before any decline in pressure is noted and that the

⁵ We are indebted to the members of the house staff of The North Carolina Baptist Hospital, and particularly to Dr. William L. Venning, for their cooperation in following the blood pressure of these patients.

tis. This patient was under observation continuously in the hospital for a period of sixteen months. Because of the more complete and convincing nature of the results obtained on this patient, the details of the experiment are confined to this one case. After a control period of observation from June 1 to July 7, 1943, the

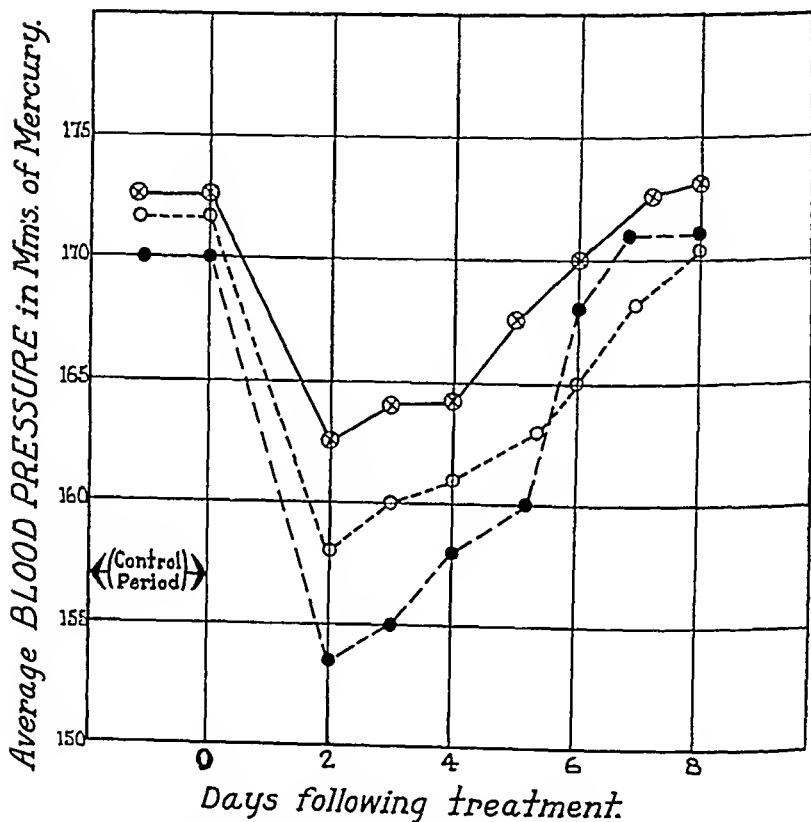


FIG. 1. THE EFFECT OF TUNG OIL ON THE BLOOD PRESSURE OF THE HYPERTENSIVE RAT. Curves showing the effect of administering 12 grams of refined tung oil (⊗—⊗), the same amount of oxidized oil (●—●), and the aqueous solution obtained by adsorption and elution of the active principle (○—○), to groups of six animals. Each reading is the average of the observed mean blood pressures. After a control period, the animals were fed the material under investigation mixed with their food, at the time designated on the abscissa as 0.

patient was treated with an active oxidized marine oil in doses of 15 cc every two hours from July 7 to September 7, 1943. He was then given an equal dose of an inactive oil as a placebo during the period from September 7, 1943 to February 28, 1944, at which time the administration of active oil was resumed and continued until July 1, 1944, when a placebo was again administered until the

active principle is water soluble and dialyzable as in the case of the orally effective principle in renal extracts (5).

The question arises as to whether the observed lowering of the blood pressure is a beneficent effect, as would be expected if this form of therapy is supplying a deficiency induced by injury of the kidney (9). Insofar as our observations on the human are concerned, there is no evidence of a detrimental effect as determined by the subjective feelings and objective tests of renal function when the blood pressure is markedly reduced. In the rat, also, preliminary studies (with Dr. Francis Reichsman) indicate an actual increase in survival time of animals maintained on active oils, compared to untreated controls.

From a practical therapeutic standpoint it is necessary to emphasize that excessively large amounts of material are necessary to induce a demonstrable effect in the human. Hence, although of great theoretical interest, we feel that the use of oils, as well as renal extracts, must still be considered as purely experimental. Until a more readily available source of large amounts of the active principle becomes available, it is impractical to attempt the treatment of patients except on an experimental basis.

SUMMARY

A number of refined oils derived from marine fishes when administered orally are effective in reducing the blood pressure in experimentally induced hypertension in laboratory animals (rats, dogs). Comparable effects were elicited in three human patients suffering from hypertension.

Among oils of vegetable and animal origin, only tung oil was found to have an effect in reducing the blood pressure in hypertensive animals.

Oxidation enhances the activity of certain oils; saponification prior to oxidation in some cases further increases their effectiveness. The blood pressure reducing capacity of the oils is independent of their original vitamin A content, and is retained after destruction of this vitamin by oxidation.

Fractional separation of the oil by distillation *in vacuo*, freezing, or distribution between aqueous organic solvents, partially effects a concentration of the effective blood pressure reducing agent. By adsorption on a synthetic resin and desorption with aqueous alkali, it is possible to obtain the active principle in aqueous solution.

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minimum values are found several weeks after cessation of therapy. The return of the blood pressure to the pretreatment level while the patient was receiving a placebo is also delayed. In general, therefore, the response is similar to that observed in the smaller experimental animal (rat, dog) except for the more gradual change with time, as is to be anticipated.

CONCENTRATION OF THE ACTIVE PRINCIPLE IN AN AQUEOUS MEDIUM. Although the oxidized marine oils are sufficiently palatable to permit their direct use in the hypertensive patient and experimental animal, it was desirable to concentrate the active principle and obviate the necessity of feeding large amounts of inert oil.

Distillation, as we have already seen, is not uniform in its effects, nor does it effect the desired concentration. Steam distillation also fails to remove the active principle. Fractional freezing was equally unsatisfactory, although in some oils it was possible to concentrate the active principle two or three fold by this method. The extraction of the oils, dissolved in ten volumes of petroleum (skellysolve) or ethyl ether, by eighty percent aqueous ethyl alcohol was laborious and, although effecting a considerable degree of concentration, was discarded for the following procedure:

Trial of a number of adsorption agents (charcoal, kieselguhr, alumina, diatomaceous earth, etc.) led to the adoption of a commercially available synthetic resinous ion-exchanger (amberlite I R 100) as the most convenient and effective. When this adsorbant was agitated with the oil in a ratio of about one part (by weight) of the adsorbant to twenty parts of oil, the active principle was removed from the oil. After filtering the oil through cloth, the adsorbant was placed in a continuous extraction apparatus arranged as in the ordinary Soxhlet extractor and treated with low-boiling skellysolve until free of adherent oil. The adsorbant was then dried in the air with the aid of a fan and stirred with two percent aqueous sodium or potassium hydroxide solution. After filtration, this process was repeated three or four times. The combined filtrates are found to contain the active material in aqueous solution, and may be further concentrated by evaporation and fractional solution in aqueous ethyl alcohol or other water-soluble organic solvents.

DISCUSSION. The results of the present study demonstrate the capacity of certain marine and vegetable oils to reduce the blood pressure of experimental animals and human patients with chronic hypertension. The same materials do not affect the pressure of normal animals. The question arises as to the relation of the active principle responsible for this action to that present in renal extracts. Insofar as the available facts are concerned, the results obtained by extracts of these two sources are comparable and it is possible that we are dealing with the same substance or substances of related chemical structure. The active principle present in oils is apparently a derivative obtained by oxidation of a fatty acid or other esterifiable substance, since saponification prior to oxidation in many cases increases the yield of the active material. The fact that auto-oxidation and the use of relatively feeble oxidation agents results in the formation of active extracts suggest that the precursor concerned is unsaturated. The resulting

very rapidly as illustrated in figure 1. It was found that single intravenous injections of 120 mgm./kgm. of body weight produced an average plasma concentration of 36 ± 6 mgm./100 cc. five minutes following administration of the compound to dogs. Within ninety minutes thereafter the plasma concentration fell to 2.0 to 3.6 mgm./100 cc. and within three hours less than 1 mgm./100 cc. was present.

Since for kidney function tests and for therapeutic use it was desirable to maintain given concentrations of the agent in the blood stream, other modes of administration were investigated.

The relationship of mode of administration to the maintenance of PAH plasma concentration is summarized in figure 2. The modes of administration studied were oral (by stomach tube), subcutaneous, intramuscular and intravenous.

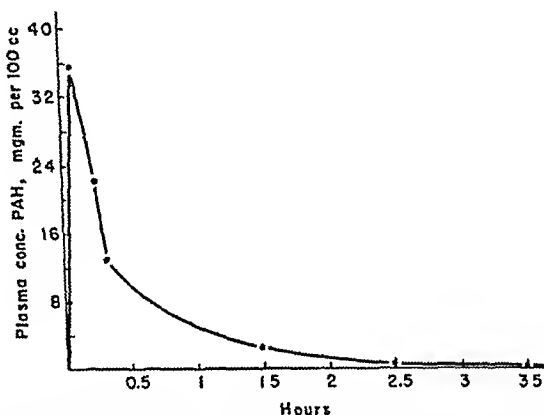


FIG 1 Illustrating the rapid fall in plasma concentration of p-aminohippuric acid following the intravenous administration of a 120 mgm./kgm. dose of the compound. The coordinates represent the average of data on 4 dogs

Uniformly, the dogs were given 200 mgm./kgm. of PAH by the mode of election, followed 15 minutes later by an intravenous injection of 120 mgm./kgm. of PAH in order to attain rapidly the desired plasma concentration. At hourly intervals for 7 hours the initial dosage was repeated except for the constant intravenous injection where the initial dose was 120 mgm./kgm. and the rate of infusion was 120 mgm./kgm./hr. over a period of 8 hours.

It is apparent that a predetermined plasma concentration of PAH (about 30 mgm./100 cc. in this instance) could be maintained by intravenous, subcutaneous and intramuscular modes of injection, but that the oral administration of PAH was ineffective in maintaining a satisfactory plasma concentration of the drug. Similarly, oral administration of PAH to mice (7) was ineffective in producing or maintaining a high plasma concentration of PAH.

There were practical drawbacks to both the subcutaneous and the intramuscular modes of administration of the agent. The injection of a 20 per cent solution

PARA AMINOHIPPURIC ACID; ITS PHARMACODYNAMIC ACTIONS

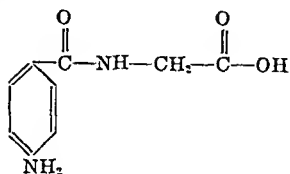
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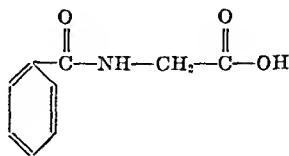
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This research was undertaken when it became apparent from the work of Smith and his associates (1), Bing (2), Lauson, Bradley and Cournand (3), Selkurt (4), and Warren, Brannon and Merrill (5) that sodium p-aminohippurate was satisfactory for the clinical and experimental estimation of effective renal plasma flow and tubular excretion, and when we found that the compound was useful for the suppression of penicillin excretion (6). In the determination of renal tubular excretion, fairly high plasma concentrations of the drug are maintained for short time intervals, and in the inhibition of penicillin excretion lower plasma concentrations of sodium p-aminohippurate (PAH) are maintained over a period of days, as in the treatment of sub-acute bacterial endocarditis, for example. This dual nature of the use of PAH, as a kidney function test agent and also as an adjuvant to penicillin therapy has necessitated a comprehensive study of the distribution, inactivation, elimination and toxicity of this agent. The pharmacological studies of this compound form the subject of this report. The toxicity of the agent is reported in an accompanying article (7).

Physical and chemical characteristics of p-aminohippuric acid. The compound is of a colorless crystalline nature, melting at 194-5°C., and is freely soluble in water as the sodium salt. Solutions of the sodium salt can be prepared easily by adjusting a given concentration of the acid to neutrality with sodium hydroxide. The molecular weight of the acid is 194.09. As will be noted from the name and chemical structure, the compound is a derivative of hippuric acid which is a metabolite formed in the body by the conjugation of benzoic acid with glycine.



p-aminohippuric acid



hippuric acid

The compound may be determined readily quantitatively in body fluids by a modification of the conventional Bratton and Marshall test for sulfonamides as described by Goldring and Chasis (8).

Plasma concentration of PAH following single intravenous injections. This mode of administration was used initially to attain quickly a high plasma concentration of PAH. However, the concentration of the compound in the blood stream fell

Recalling the structure of the compound, it seemed appropriate to examine the enzymatic hydrolysis of PAH and the absorption and excretion of p-aminobenzoic acid (PAB), for the latter compound would be formed as a result of hydrolysis of the peptide linkage of PAH in the presence of gastric or intestinal enzymes. While an exhaustive study of the possibility of the enzymatic splitting of PAH was not conducted, the compound was incubated overnight at 37.5°C. with a commercial (Difco) trypsin, which also contained some carboxypolypeptidase. PAH

TABLE 1
The progressive percentage recovery of a 120 mgm./kgm. intravenous dose of p-aminohippuric acid from the urine of dogs

DOG	PAH RECOVERED, PERCENTAGE OF DOSE ADMINISTERED						
	0-1 hour	1-2 hours	2-3 hours	3-4 hours	4-22 hours	22-30 hours	30-48 hours
58	43.3	63.7	71.5	74.2	77.9	77.9	78.1
84	62.0	78.7	86.3	87.8	90.1	91.7	92.1
85	69.7	71.8	75.2	76.5	78.4	78.4	78.5
88	56.1	69.1	73.6	75.4	78.0	78.2	78.2
Average....	55.3	70.8	76.7	78.5	81.1	81.6	81.9

TABLE 2
The percentage urinary and fecal recovery (at the end of 48 hrs.) of 200 mgm./kgm. doses of p-aminohippuric acid and p-aminobenzoic acid administered orally to dogs

DOG NO.	DOSE	p-aminohippuric acid		
		% RECOVERED IN URINE	% RECOVERED IN FECES	% UNACCOUNTED FOR
31	200 mgm./kgm.			
55	1.94			
56	2.06	41.0	4.1	54.9
60	2.50	29.7	13.4	56.9
	2.56	20.9	6.6	72.5
		35.2	4.6	60.2
p-aminobenzoic acid				
51	1.94	62.0	2.0	36.0
55	2.10	70.3	1.5	28.2

was not hydrolyzed in a number of experiments, as judged by the lack of any change in amino nitrogen. The plasma concentration, urinary elimination, and fecal elimination of PAB following oral administration to dogs in the same dosage as PAH were studied in spite of the *in vitro* evidence that PAH was not hydrolyzed enzymatically in the gastrointestinal tract. The results of two such experiments are presented in table 2. The evidence supports the conclusion that this compound was absorbed readily and for the most part was excreted in the urine.

The modes of renal elimination of both PAH and PAB have been studied. A

was irritating because of its hypertonicity. Thus the sites of injection became painful and swollen. One could not avoid this effect unless the solution was diluted, whereupon the volume of material injected became a detrimental factor. Also, the frequency of injection necessary to maintain a uniform plasma concentration detracted from the merit or convenience of the procedure. Thus, of the modes, the intravenous infusion of the material appeared to be the most desirable and has proved very satisfactory and convenient in both laboratory (6 a-c) and clinical experience (6-d) where the infusions have been maintained for 30 to 54 hours.

The results obtained with the oral administration of PAH led us to study the fate of the compound when administered in this, and the parenteral, manner.

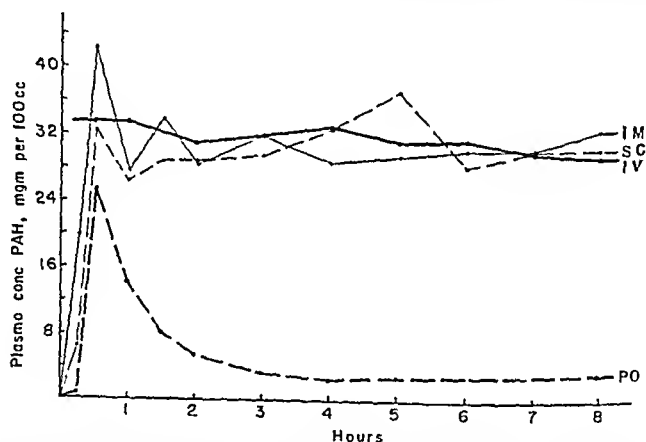


FIG 2 Demonstrating the plasma level of p aminohippuric acid in response to oral (p o), intravenous (i v), subcutaneous (s c) and intramuscular (i m) routes of administration of the compound

The fate of PAH in the body. The extreme rapidity with which intravenously injected PAH appears in the urine is illustrated by the data in Table 1. In this experiment 4 dogs were given 120 mgm./kgm. doses of the compound and the urine was collected at predetermined intervals thereafter for 48 hours. From the data it can be calculated that 43.3 to 69.7 per cent of the material recovered was excreted within the first hour after the injection. The total amount of PAH which appeared in the urine was 78.1 to 92.1 per cent (average 81.9 per cent) of the amount administered.

The observation that one could not maintain high plasma concentrations of PAH when the material was administered by stomach tube led to studies designed to elucidate the cause. Each of four dogs was given 200 mgm. of PAH/kgm. Their urine and feces were collected over a period of 48 hours and were analyzed for PAH. It appears from the data in Table 2 that only a small amount of the compound appeared in either feces or urine and that from 54.9 to 72.5 per cent of the material was unaccounted for

A very large part of the PAB that is filtered through the glomerulus is reabsorbed by the renal tubules. A most interesting observation with regard to the PAB clearance is one first pointed out by Smith, Finkelstein and Aliminos (1-b) and substantiated in part by the data in Table 3. It is their belief that the rise in the clearance of PAB is due to its conjugation with glycine in a manner analogous to the formation of hippuric acid in the body. If such a conjugation occurred there would be superimposed on the basal PAB clearance the increasing tubular excretion of PAH as it was formed. They report an experiment wherein the clearance ratio finally exceeded 1.0 sometime after the PAB was injected.

The mode of renal elimination of PAH has been shown to be both by glomerular filtration and by renal tubular excretion (1). Because of its high rate of excretion by the renal tubular epithelium the compound can be used to determine both renal plasma flow and also the functional capacity of tubular excretion (T_m). The fact that both PAH and penicillin compete for excretion by the same tubular mechanism has made it possible to prolong the maintenance of penicillin in the body by the simultaneous administration of PAH, thus decreasing the rate of excretion of penicillin (6).

Goldring and Chasis have presented in their recent publication (8) a clear and concise description of the procedures for and interpretation of the use of PAH in the determination of T_m (maximal tubular excretory capacity) and effective renal plasma flow. Actual protocols and forms for recording the results of the tests using PAH are to be found in Appendices D and E of their book. They found that the T_m of PAH in their series of normal adult patients was 76.1 mgm./min.

Our own experience with the use of PAH as a renal function test suggests that two important points in the procedure bear reiteration. For the measurement of T_m in man the plasma PAH concentration should be between 60 and 80 mgm./100 cc. and for the measurement of minimal renal plasma flow it should be about 2 mgm./100 cc. In dogs a PAH plasma concentration of 40 to 50 mgm./100 cc. is sufficient for the measurement of T_m , and for the determination of minimal renal plasma flow the PAH plasma concentration should be less than 2 mgm./100 cc.

Conjugation of PAH was studied using bilaterally nephrectomized rats. The operation was done in two stages with sufficient time (over one week) intervening between the two nephrectomies to permit the animals to recover from the initial operation. The second stage was performed in the late afternoon and the rats were permitted to recover overnight from the procedure. This routine minimized the effects of the operations and yielded animals which usually lived about 96 hours following the last surgery.

The morning following the operation the animals were injected intraperitoneally with 0.5 gm. of PAH/kgm. Analyses for free and total PAH were carried out periodically thereafter for 24 hours on blood obtained from the tail. The results of these experiments are graphed in figure 3.

From the graphs it may be concluded that PAH can be conjugated in the body of the rat. At the end of 24 hours about 27.4 per cent of the total amount of the drug in the body of the animals exists in this form under these conditions. It

number of experiments were performed in which the compounds were administered orally, followed one hour later by a series of ten minute renal clearance periods. Protocols on two such experiments are included in table 3. Several points of interest stand out in these data.

The plasma concentrations of the two drugs in these experiments are indicative in a measure of their relative absorption from the digestive tract. At the same dosage level the plasma concentration did not rise above 0.9 mgm./100 cc. for PAH whereas the PAB plasma concentration was 39.9 mgm./100 cc. This is not

TABLE 3

Protocols of two experiments wherein the renal clearance of p-aminohippuric or p-aminobenzoic acid was measured at alternate ten minute intervals beginning one hour after the oral administration of 200 mgm./kgm. of either compound to a dog

Six hundred cubic centimeters of water was given orally one to one and one half hours before the compounds were given. One half hour before the clearances were begun an intravenous infusion of 5 per cent glucose solution was started which continued at a rate of 4.5 to 5 cc./min. for the duration of the experiment.

TIME ¹	PLASMA ² CONC.	CREATININE CLEARANCE	CLEARANCE OF COMPOUND	CLEARANCE RATIO	FILTRATION FRACTION	URINE FLOW
p-aminohippurate (PAH)						
hr.:min.	mgm./100 cc.	cc./min.	cc./min.			cc./min.
1:00-1:10	0.7	92.1	231.9	2.5	0.40	6.60
1:20-1:30	0.7	79.2	226.7	2.9	0.35	5.90
1:40-1:50	0.8	77.5	197.9	2.6	0.39	4.90
2:00-2:10	0.9	73.8	179.4	2.4	0.41	3.90
2:20-2:30	0.8	73.8	197.1	2.7	0.37	4.25
2:40-2:50	0.8	80.5	193.3	2.4	0.42	4.38
p-aminobenzoate (PAB)						
1:00-1:10	39.9	63.9	10.0	0.16		4.15
1:20-1:30	39.9	66.1	11.1	0.17		3.75
1:40-1:50	39.2	68.9	13.6	0.20		3.55
2:00-2:10	37.9	64.6	15.1	0.23		3.75
2:20-2:30	36.3	77.4	17.8	0.23		4.65

¹ The experiment was begun one hour after the compound was administered.

² Not corrected for binding.

a true index of their relative rates of absorption since the clearance of PAH was roughly 15 to 20 times that of PAB in these tests. It is also unlikely that any considerable amount of PAB was formed in the intestinal tract from PAH and excreted, because of the high renal clearance in these experiments which is typical for PAH.

These results indicate that, at least for experimental purposes, the oral administration of PAH for the determination of renal plasma flow is quite practical since very low but uniform plasma concentrations are ideal for such studies. This mode of administration for clinical use might be feasible where only renal plasma flow information is desired.

able usefulness. It should be readily apparent that if PAH were to act like p-aminobenzoic acid (PAB) and certain local anesthetics having a p-amino group, i.e., procaine (9), in inhibiting the action of sulfonamides on organisms, this property would negate such an advantage as might accrue to the combined use of the two forms of therapy.

The effect of PAH and p-aminobenzoic acid on the growth of a strain of *E. coli* in bacto-peptone broth with and without the addition of sulfanilamide was determined. Figure 4 summarizes the data obtained in the course of such an experiment. From these results it may be concluded that: 1) except at the highest concentrations neither PAH nor PAB influenced the growth of *E. coli*

TABLE 4

The concentration of p-aminohippuric acid in whole blood and plasma of dogs five and ninety minutes following the intravenous administration of a 120 mgm./kgm. dose

DOG NO	MGM PAH/100 CC WHOLE BLOOD	MGM PAH/100 CC PLASMA	BLOOD CONC. PLASMA CONC.
5 minutes after injection (120 mgm./kgm.)			
10S	22.8	29.2	.78
112	28.4	36.8	.77
30	30.4	41.6	.73
D-7	26.4	36.0	.74
111	21.6	30.0	.72
Average	25.9	34.7	.75
90 minutes after injection			
10S	1.9	2.6	.74
112	1.7	2.2	.77
30	1.3	1.9	.68
D-7	1.0	1.5	.67
111	1.8	2.4	.75
Average	1.5	2.1	.72

as indicated by turbidity readings at the end of a comparable period of incubation; 2) M/200 sulfanilamide almost completely inhibited the growth of the organisms; 3) p-aminobenzoic acid almost completely inhibited the bacteriostatic effect of sulfanilamide, and this effect was still definitely present at the highest dilution (M 128,000) of PAB; 4) the inclusion of four times as much PAH as sulfanilamide, on a molar basis, did not influence the bacteriostatic activity of sulfanilamide.

Since PAH does not antagonize the bacteriostatic effect of sulfonamides, and since none of the latter primary amino compounds commonly used in clinical medicine is excreted by the renal tubules, it seems unlikely that p-aminohippuric acid would either interfere with the activity or increase the toxicity of such compounds if administered to a patient also receiving penicillin. However, one

can be calculated from the values given for the ordinates that the total amount of PAH in the blood decreased during the course of the experiments, which may be due to any or all of a number of causes.

Estimations of the degree of binding of PAH on plasma proteins of both dogs and humans were carried out *in vitro* by a dialysis procedure. We were not able to establish reproducible values for the binding of this compound since it was so poorly bound that the values obtained approached the expected error of the method. Smith and his associates (1-b) have recommended a correction of 0.83 to calculate the amount of PAH free in plasma water, and this includes a correction for binding. It is our opinion that this is quite adequate since our values for plasma binding have been considerably less than 10 per cent except for an occasional sample. We have not used a correction for binding in our data.

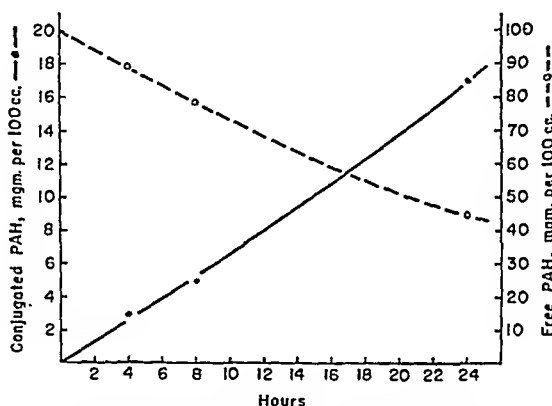


FIG. 3. Demonstrating the average acetylation of p-aminohippuric acid by two-stage bilaterally nephrectomized rats administered 0.5 gram/kg. of the compound intraperitoneally.

The distribution of PAH in blood was studied in five dogs. The purpose was to determine roughly whether the compound was distributed equally between cells and plasma and to give some indication of how rapidly the distribution took place. These data are summarized in Table 4. While differences in hematocrit values for the dogs probably accounts for the variation in the blood/plasma concentration ratios in these data, one may conclude that some seventy-five per cent of the drug which is in the blood stream is in the plasma and that the equilibration takes place within five minutes following its injection. It is probably reasonable to attribute the slight differences in the ratios for the two samples from a given dog as being due to error in the analyses at the low plasma concentrations.

The effect of PAH on sulfonamide activity was studied since the latter group of compounds is being used by some in conjunction with penicillin; this is true especially in subacute bacterial endocarditis, a disease for the treatment of which it is anticipated that the combined use of PAH and penicillin will find consider-

cent of the drug unaccounted for. That the compound was not hydrolyzed to any notable extent in the gastrointestinal tract was indicated by the following information: 1) trypsin and carboxypolypeptidases did not hydrolyze the compound *in vitro*. 2) If this hydrolysis had occurred *in vivo* the p-aminobenzoic acid formed thereby would have been rapidly absorbed resulting in a relatively high plasma concentration of the aromatic amino compounds and an overall renal clearance lower than that of PAH at any plasma concentration. As was actually the case only low plasma concentrations of PAH occurred following its oral administration. Renal clearance data obtained in such experiments supported the conclusion that all the diazotizable material was probably PAH. The fact that the dogs did not conjugate the material precluded that as a mode of alteration of the molecule.

Our experiments indicated that very little if any PAH was bound on plasma proteins. While equilibration of the material between the blood cellular elements and plasma occurred quickly, approximately 75 per cent of the drug remained in the plasma. The rat was capable of conjugating PAH, but this occurred more slowly than was the case for sulfathiazole, sulfamethazine, sulfadiazine or sulfamerazine tested similarly in this laboratory (10). It is likely that this information is more directly referable to the human than is the similar finding in dogs.

Our experiments substantiate the conclusions of others that sodium p-aminohippurate can be used satisfactorily to measure T_m and renal plasma flow.

PAH did not antagonize the bacteriostatic activity of sulfanilamide even though one of its components is p-aminobenzoic acid which characteristically has that property. This observation has a very material bearing on the combined use of sulfonamides with penicillin and PAH in highly refractory diseases of bacterial origin.

The toxicological aspects of this problem are being reported in a separate communication (7).

Acknowledgment: The diversity of the methods used in such an evaluation of a compound has made it desirable to elicit the assistance of other members of the department. Among these Dr. A. Kathrine Miller was responsible for the conduct of the PAH-sulfonamide antagonism experiments, Mr. Kenneth L. Sprague prepared the nephrectomized rats and Miss Eleanor Weiss of the Department of Biochemistry performed the experiments involving the enzymatic digestion of PAH.

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should be reminded that both PAH and sulfonamides respond to the same colorimetric tests and that plasma determinations presumably for one or the other compound under these conditions would be misleadingly high unless this additive effect were appreciated.

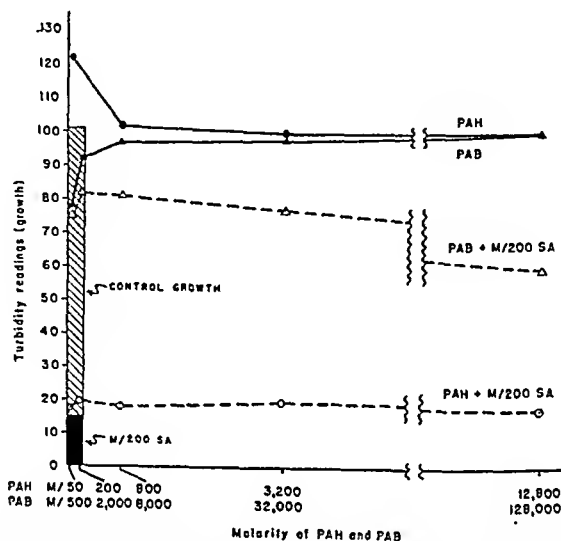


FIG. 4. The effect of p-aminohippurate (PAH) and p-aminobenzoate (PAB) on the growth of *E. coli* in a bacto-peptone broth and on the bacteriostatic action of sulfanilamide (SA). The figure illustrates that neither PAH nor PAB influenced the extent of growth except at the higher molarities. M/200 SA almost completely inhibited growth of the inoculum. This bacteriostatic effect of SA was not inhibited to any notable extent by M/50 PAH whereas approximately 60 percent inhibition occurred in the presence of M/128,000 PAB.

SUMMARY

The findings recorded in this report may be summarized briefly:

Following single intravenous injections of sodium p-aminohippurate its plasma concentration fell very rapidly (from about 36 to 2 mgm./100 cc. in 90 minutes). This was due principally to distribution and excretion of the compound. Since PAH was excreted by the renal tubules in addition to glomerular filtration, 43.3 to 69.7 per cent of the amount administered intravenously appeared in the urine within the first hour thereafter.

In order to maintain a uniform plasma concentration over a long period of time the preferable mode of administration was by venodysis, although both the subcutaneous and intramuscular routes were used for this purpose. The oral route of administration neither gave nor maintained adequate plasma concentrations of the compound, except for the determination of renal plasma flow.

Administered orally, 20.9 to 41.0 per cent of a dose of PAH was excreted in the urine and only 4.1 to 13.4 per cent appeared in the feces, leaving 54.9 to 72.5 per

TOXICOLOGICAL MANIFESTATIONS AND PATHOLOGICAL FINDINGS FOLLOWING THE ADMINISTRATION OF PARA AMINOHIPPURIC ACID

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A previous series of papers (1-4) reported that when sodium p-amino hippurate was administered intravenously together with penicillin the former compound competed with the antibiotic agent for a common renal tubular transport mechanism. By this means a high penicillin plasma concentration could be maintained for prolonged periods of time. Since it was anticipated that combined therapy of this nature might be useful in the prolonged treatment of such a condition as subacute bacterial endocarditis comprehensive pathologic examinations accompanying the previously reported experiments and more extensive toxicologic evaluations seemed of paramount importance.

In an accompanying article (5) we have discussed the absorption, distribution, plasma binding, conjugation, and renal elimination of the sodium salt of p-aminohippuric acid (PAH). The toxicological studies of this compound are presented as a separate communication in order that the description of the overall research shall not become burdensome.

The purpose of this report, then, is to present the results of our investigations of the acute and subacute toxicity of p-aminohippuric acid for several species of animal. The procedure used, and the pathology resulting from the constant intravenous administration of PAH for periods of 48 to 54 hours were complicated by barbiturate hypnosis, the infusion of excessive amounts of fluid and the presence of penicillin. However, these experiments have been controlled, as far as possible, by similar ones in which one or two of these factors were eliminated and the others duplicated, thus enabling us to evaluate better the toxicity of the compound *per se*.

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following the last dose had a PAH plasma concentration of 6.8 mgm./100 cc. None of these animals demonstrated any toxic manifestations during the test.

Ten white mice (Carworth CF₁) were given PAH in an oral dose of 10 gm./kgm. administered as a 20 per cent aqueous solution (pH 6.8). No toxic manifestations, other than the temporary discomfort from such a large dose, were seen at any time.

In order to secure data for acutely toxic doses of this compound, 120 white mice (Carworth CF₁) weighing between 15 and 20 gm. were injected intravenously with various doses of a 20 per cent solution of PAH. The maximal dose was 0.75 cc. of the 20 per cent solution per 20 gm. of mouse. The rate of injection remained constant at 1 cc. per minute. The data presented in Table 1 were treated by Epstein's method (6) and the $LD_{50} \pm 2$ standard errors was calculated to be 4.93 ± 0.8 gm. per kgm. All deaths were immediate, being preceded by clonic spasms and respiratory paralysis. Some of the animals given the higher sublethal doses showed spastic paralysis of the hind quarters and gasping respiration from which they recovered.

Twenty mice were given 5.0 gm. per kgm. of 20 per cent PAH intravenously, the approximate LD_{50} . Fifteen minutes following the injections the average concentration of PAH (whole blood) in the surviving 10 mice was 642 mgm./100 cc. with a range from 428 mgm./100 cc. to 832 mgm./100 cc. One hour following the injection of a similar dose to 25 mice the 10 surviving mice had an average PAH concentration of 203 mgm./100 cc. of whole blood, with a minimum of 88 and a maximum of 480 mgm./100 cc.

In order to compare PAH with a structurally related, normal body metabolite, hippuric acid was administered intravenously to white mice as a 20 per cent aqueous solution of the sodium salt (pH 7.4). The data presented in Table 1 indicate that sodium para-aminohippurate is no more toxic than sodium hippurate when injected intravenously into white mice. The LD_{50} of the latter compound, calculated by Epstein's method, was found to be 4.15 ± 0.68 gm./kgm.

Since PAH is conjugated to some extent (5) the sodium salt of p-acetylaminohippuric acid monohydrate was injected intravenously into white mice (CF₁) following the same procedures as were used with the parent compound. The LD_{50} (table 1) was calculated to be 5.25 ± 0.52 gm./kgm. (Epstein). These data indicate that the para acetyl-amino derivative was no more toxic than the parent compound under similar circumstances.

Rabbits: PAH has been administered intravenously to seven apparently normal rabbits in an effort to secure toxic manifestations. Two preliminary experiments demonstrated that these animals would tolerate large intravenous doses. One animal was given 3.0 gm./kgm. of a 20 per cent solution of PAH into the marginal vein of the ear at a rate of 1 cc. per minute. This animal demonstrated no toxic symptoms and survived the experiment. A second rabbit similarly was given 4.0 gm./kgm. of the same solution and survived without toxic manifestations, even though a plasma concentration of 648 mgm./100 cc. was demonstrated 30 minutes following the injection.

Since infusion into the marginal vein of the ear did not permit long continued injections, the left femoral vein was chosen for the remaining experiments. The vein was exposed under local anesthesia and cannulated with a #8 woven silk catheter attached to a 21 gauge needle. By this method constant intravenous infusions could be instituted without danger of dislodging the catheter if the animal struggled or convulsions occurred. Concentrations of PAH in the blood of these animals were determined at certain intervals, and in five experiments all urine was collected during the infusion period.

TABLE 1

Mortality following the intravenous administration of solutions of the sodium salts of para-aminohippuric acid, p-acetylaminohippuric acid and hippuric acid to white mice

PARA-AMINOHIPPURIC ACID			PARA-ACETYLAMINOHIPPURIC ACID			HIPPURIC ACID		
Dose	No. of animals	No. of deaths	Dose	No. of animals	No. of deaths	Dose	No. of animals	No. of deaths
gm /kgm.			gm /kgm.			gm /kgm.		
3.25	10	0	4.00	10	0	3.50	10	0
3.50	10	1	4.50	10	2	3.75	10	5
3.75	10	1	5.00	10	3	4.00	10	4
4.00	10	2	5.50	10	6	4.25	10	6
4.25	10	0	6.00	10	9	4.50	10	8
4.50	10	5	6.50	10	10	4.75	10	9
4.75	10	6				5.00	10	7
5.00	10	4				5.25	10	10
5.25	10	6				5.50	10	10
5.50	10	4						
5.75	10	9						
6.00	10	10						

TABLE 2

Concentration of para-aminohippuric acid (in mgm./100 cc.) in the blood of rabbits following the constant intravenous infusion of sodium para-aminohippurate (10 per cent)

ANIMAL NO.	WEIGHT	RATE OF INFUSION	TOTAL DOSE	BLOOD CONCENTRATION, HOURS AFTER ADMINISTRATION						REMARKS
				$\frac{1}{2}$	$\frac{1}{4}$	1	2	4	6	
	kgm	gm /hr	gm.							
01	1.5	3.2	26.4				330	580	328	died overnight
02	2.2	2.2	28.8		130	206	264	360	516	died overnight
03	2.4	2.5	36.0	140	196	248	308	468	1088	died at 6 hours
04	2.4	2.5	33.0	174	250	280	336	368	216	died overnight
05	2.0	3.0	36.0	156	226	272	336	428	872	died at 6½ hours

By maintaining rates of infusion between 2.2 and 3.0 gm./kgm. per hour we were able to secure very high blood concentrations (table 2). Two of the animals died during the 6 hour experiments, one (#03) at 6 hours and one (#05) at 6½ hours after venoclysis was begun. Both animals were autopsied at once. The most prominent features were the marked engorgement of the visceral blood vessels and edema. The histological findings in both animals were similar

and consisted of toxic nephrosis, vascular congestion and hydropic degeneration of the liver, generalized edema, and agonal thrombosis. The remaining animals showed no signs of toxicity during the course of the experiments. All animals were prostrate when taken from the table, but though they had recovered their normal reflexes three or four hours later, each animal died during the night. Autolytic changes were such as to preclude examination of the tissues histologically, but the impression gained from gross examinations indicated that a similar picture of widespread vascular congestion and edema would have been found.

It is interesting to note that the two animals who died during the course of the experiments had the highest blood concentrations of PAH (table 2). However, it is difficult to correlate blood concentrations attained with toxicity of the compound *per se*, since one rabbit whose blood had a concentration of 648 mgm./100 cc. survived whereas another with a maximum concentration of 368 mgm./100 cc. died overnight.

TABLE 3

The urinary excretion of p-aminohippuric acid for rabbits following the constant intravenous infusion of sodium p-aminohippurate (10 per cent)

ANIMAL	WEIGHT	TOTAL DOSE GIVEN DURING FIRST 4 HOURS	TOTAL DOSE GIVEN DURING 6 HOURS	AMOUNT EXCRETED		PERCENTAGE OF DOSE EXCRETED	
				During first 4 hours	During 6 hours	During first 4 hours	During 6 hours
	kgm.	gm.	gm.	gm.	gm.	per cent	per cent
01	1.5	19.2	26.4	4.75	7.11	25	27
02	2.2	19.2	28.8	12.74	19.37	66	67
03	2.4	24.0	36.0	11.31	16.64	47	46
04	2.4	21.6*	26.4	14.04	18.35	65	70
05	2.0	21.6*	36.0	16.68	21.19	77	59

* Dose given during first 4½ hours.

Urine samples from these animals were collected by catheterization during the period of infusion and the amounts of PAH excreted during these experiments were determined (table 3). Examination of the data demonstrates the rapidity with which the compound was excreted. An average of 56 per cent of the amount administered during the first four hours was excreted during the same time interval. An average of 54 per cent of the total dose given was excreted during the first six hours.

Short term infusion experiments in dogs: In an attempt to provoke toxic manifestations and eventual death we resorted to constant intravenous administration of large amounts of PAH to four normal dogs for periods ranging from 2.5 to 8 hours. One additional animal was anesthetized and the renal vessels were ligated bilaterally before injecting PAH.

The animal was placed on its back in a cradle, lightly restrained, catheterized and the urine allowed to drain. One femoral vein was exposed under local anesthesia and was cannulated by means of a woven silk catheter attached to a 21 gauge needle. The needle was

connected to either an electric constant rate infusion apparatus or to a Murphy drip apparatus containing the infusion fluid. The rate of infusion varied from 250 mgm./kgm./hr. to 3.3 gm./kgm./hr., depending on the purpose of the experiment. The concentration of PAH was varied also from 2.5 per cent to 40 per cent, the usual concentration employed being 20 per cent. Blood samples were drawn periodically for PAH determinations and hematological studies.

The most striking manifestation of toxicity seen during the infusion of PAH to dogs was the occurrence of convulsions. These usually did not occur until plasma concentrations approximating 600 mgm./100 cc. were attained. Dog #555 attained a plasma concentration of 611 mgm./100 cc. 5½ hours after beginning the injection and did not demonstrate any toxic signs, whereas a second dog (#BW) began convulsing at 1¼ hours, just after a plasma concentration of 639 mgm./100 cc. had been reached. Another animal (B) on the first day after receiving a total dose of 7.4 gm. of PAH/kgm. during 5 hours (table 4) attained a maximal plasma concentration of 404 mgm./100 cc. without convulsions. On the following day a maximal plasma concentration of 591 mgm./100 cc. was reached two hours after venoclysis was started; convulsions, however, did not occur until one and one-half hours later (3½ hours after beginning venoclysis) when the plasma level had decreased to 450 mgm./100 cc. The fourth normal dog (H) received a total dose of 130.98 gm. of PAH during a period of 7 hours, equivalent to 9.85 gm./kgm. No signs of toxicity other than hematuria at 6 hours were noted, even though the plasma concentrations of PAH reached a level of 491 mgm./100 cc. These findings strongly suggest that the rate at which the tremendously high plasma concentrations of PAH were reached was as important as the blood level attained in initiating toxic reactions. It would seem, then, that in these and in the acute toxicity experiments on mice and rabbits the rapid injection of very hypertonic solutions contributed in large measure to the death of the animals. Three hours following cessation of the drip the dog was removed from the table and in a few minutes had recovered sufficiently to walk back to its cage.

The hematologic studies of two dogs (H and BW) indicated an increase in the total leucocyte counts and of another (B) a decrease during the first day's experiment. In the former the differential counts revealed an increase in polymorphonuclear neutrophils and no changes in the other forms, and for dog B the differential counts remained the same. Two of the dogs showed an increase in erythrocytes, dog H 4 per cent and dog BW 21 per cent. These findings, combined with the increased hematocrits indicate the presence of hemoconcentration.

Pathologic Studies Two animals (BW and B) were autopsied immediately following their death. Histopathological examinations disclosed essentially similar findings in the two dogs.¹ The significant changes observed were generalized edema and vascular congestion, with focal necrosis of the liver,

¹ At the request of the Editor the detailed descriptions of the gross and microscopic studies on these animals and those on the long term infusion experiments have been deleted from the paper. Individuals desiring these observations may secure a copy by addressing their inquiries to this Laboratory.

TABLE 4

The plasma concentrations and toxic manifestations resulting from the constant intravenous infusion of sodium p-aminohippurate into dogs

Period in hours	DOG 555												DOG H							
	First test								Second test*											
	0 to 1	1 to 2	2 to 3	3 to 4	4 to 5	5 to 5½	5½ to 6½	6½ to 7½	7½ to 8	0 to 1	1 to 2	2 to 3	3 to 5½	0 to 1	1 to 2	2 to 3	3 to 4	4 to 5	5 to 6	6 to 7
Priming dose mgm /kgm	200	100	100	150	200	200	200	400	400	200	400	400	200	400						
Infusion rate mgm /kgm /hr	250	375	500	625	750	1000	1250	2000	3330	952	1905	2 857	2 857	771	771	771	1547	1547	1935	1935
Maximal plasma concentration mgm /100 cc	48 4	72 2	85 5	111	129	437	195	327	440	160	284	471	611	110	149	179	309	352	443	491
Total dose gm			92 5								183 5				130 98					
Total dose gm /kgm			7 7								14 5				9 85					
Remarks	Salivation, vomiting, mild tremors. Animal was returned to cage, recovered and allowed two weeks rest before using on the second test								Vomiting, strychnine like convulsions, died overnight				Hematuria at 6 hours. No other toxic manifestations. Died overnight							

Period in hours	DOG A					DOG BW							DOG B							
													First test				Second test†			
	0 to 1½	1½ to 5½	5½ to 5½	5½ to 6	6 to 6½	0 to 1	1 to 1½	1½ to 2½	2½ to 3	3 to 4	4 to 5	at death 5½	0 to 2½	2½ to 3	3½ to 5	0 to 1	1 to 2	2 to 3½	4	4½
Priming dose mgm /kgm			500	500	1000	205										200				
Infusion rate mgm /kgm /hr	526	1053	1053	1053	1053	2730	2730	2730					900	1800	1800	1800	1800	1500		
Maximal plasma concentration mgm /100 cc	213	778	901	1060	1372	520	639	835	599	552	599	556	202	222	404	334	591	450	350	325
Total dose gm		86 8						78						73 6		68				
Total dose gm /kgm		7 6						8 9						7 4		6 8				
Remarks	Dog anesthetized and nephrectomized. Died overnight. Autopsy revealed widespread vascular congestion, edema, and petechial hemorrhages in intestinal mesenteries.					Tonic convulsions began at 1½ hrs. Controlled by barbiturates. Tachycardia and tachypnea—died at 15½ hours. Autopsied.							First test Stercoraceous vomiting, decreased heart rate and arrhythmia. Dog returned to cage and 15 hrs later venoclysis was resumed. Tachycardia, arrhythmia, decreased respiration and convulsions occurred. Death at 4½ hrs. Autopsied.							

* 14 days elapsed between first test and second test

† 15 hours elapsed between completion of first test and beginning of second test

parenchymatous degeneration of the renal tubular epithelium, and occasional subendocardial hemorrhages

Long term infusion experiments. The experiments described have all been

short term procedures devised to produce very high plasma concentrations of PAH in an attempt to secure toxic manifestations. Since in the therapeutic use of this compound much lower plasma concentrations may be maintained for considerably longer periods of time, particular attention was devoted to those dogs who received the drug alone, the drug together with penicillin, or who received penicillin alone for extended periods of time. The experiments in which these dogs were used have been described in a previous paper (4), which may be referred to for PAH and penicillin plasma concentrations and renal clearance data.

In the interest of brevity the detailed gross and microscopic findings following autopsy of these animals will not be given here.² Pertinent details of the procedures followed, the doses given, the volume of fluid administered, the plasma concentrations attained and the weight changes will be given for each animal.

Dog #113. Female. Anesthetized with 30 mgm./kgm. of sodium pentobarbital intravenously and maintained with subcutaneous or intravenous doses of the same drug as needed during the 48 hour period. The trachea was intubated and the tube left in position for the entire period. An indwelling catheter was placed in the bladder under aseptic conditions, tied in place by a suprapubic approach to the urethra and attached to the apparatus for collecting urine under sterile conditions (3). One jugular and one femoral vein were exposed, the former being used for venoclysis, the latter for the collection of blood samples. An initial dose of 120 mgm./kgm. of PAH and 5000 units of penicillin was administered. A constant intravenous infusion of approximately 3 cc./min. of 1.5 per cent PAH in glucose-saline solution (0.45 per cent NaCl + 2.5 per cent glucose) was maintained for 48 hours. To this solution was added sufficient penicillin to give a rate of administration of 15 units/min. for the 48 hours. The total volume of fluid administered during 48 hours was 8.64 liters. The plasma concentrations of PAH throughout the experiment averaged 31.7 mgm./100 cc. The weight of the dog increased from 14.0 kgm. at the start of the experiment to 15.8 kgm. at its completion.

Dog #81. Female. (Control to #113). This animal was prepared in the same manner as #113 and treated similarly, except that no PAH or penicillin was given. A constant intravenous infusion of glucose-saline solution (0.45 per cent NaCl + 2.5 per cent glucose) was given at a rate of 3 cc./min. for 48 hours. The total volume of fluid given was 8.6 liters during 48 hours. The dog weighed 22 kgm. at the beginning of the experiment and obviously gained weight during the test, but since her weight exceeded the maximal scale reading of 22.5 kgm. we were unable to record the total weight gain.

Dog #39. Female. This animal was anesthetized and prepared in a manner similar to #113. Following the initial dose of PAH (120 mgm./kgm.) and penicillin (2500 units) infusion was instituted at a rate of approximately 3.0 cc./min. of a 1.5 per cent PAH solution in glucose-saline solution (0.45 per cent NaCl + 2.5 per cent glucose). After 700 cc. had been given, the amount of PAH was increased, so that the same quantity of PAH would be given in 2 cc./min. of a glucose saline solution (0.225 per cent NaCl + 3.75 per cent glucose). At a second site, 5 U./cc. of penicillin in saline and glucose was infused at a rate of 3 cc./min. After 24 hours the infusion fluid was changed to the original concentration of glucose-saline solution. The total amount of fluid given was 4.73 liters. The plasma concentration of PAH averaged 30.7 mgm./100 cc. No weight was secured on this animal.

Dog #114. Female. (Control to dog #39). This dog was anesthetized in the same manner as dog #39 and was treated similarly except that no PAH or penicillin were given at any time. This dog weighed 15.2 kgm. at the beginning of the experiment and 17.0 kgm. at its completion, 48 hours later.

² See footnote 1

Dog #63. Female. This animal was prepared as previously described except that the brachial veins of both fore-limbs were used for infusion, one for penicillin and one for PAH. Penicillin was infused at a rate of approximately 15 units/min. during the 48 hours following a priming dose of 5000 units. PAH was infused in a saline-glucose solution (3.75 per cent glucose + 0.125 per cent NaCl) in order to secure ascending plasma concentrations. During the first 18 hours no PAH was given. Following a priming dose of 120 mgm./kgm. a 2.25 per cent PAH solution was infused at a rate of 2 cc./min. from the 18th to the 21st hour. At the 21st hour, following a priming dose, a 2.0 per cent PAH solution was given at 2.0 cc./min. until the 26th hour. A priming dose of 150 mgm./kgm. of PAH was given at 26:10 hours, followed by an infusion rate of 2 cc. of 5 per cent PAH per min. This was continued until 36:10 hours at which time an additional 75 mgm./kgm. dose of PAH was administered and the infusion was changed to 2 cc./min. of 7.5 per cent PAH in 2 per cent glucose. This new infusion rate was maintained for 8 hours (44:10 hr.) at which time the administration of PAH was halted. The average plasma concentrations attained were as follows: 24.4 mgm./100 cc. from 18 to 21 hours, 37.3 mgm./100 cc. from 21 to 26 hours, 71.3 mgm./100 cc. from 26 to 36 hours, 104.4 mg./100 cc. from 36 to 44 hours. The plasma concentration had decreased to 18.3 mgm./100 cc. at the 48th hour when the experiment was terminated. This animal's weight increased from 15.6 kgm., initially, to 17.2 kgm. at the completion of the experiment. Approximately 5.1 liters of fluid were infused during the 48 hours.

Dog #86. Female. The preparation of the animal was similar to that described for #63. Following a priming dose of 2500 units of penicillin, penicillin was infused at a rate of 1 cc./min. (15 units/min.) with 4 per cent glucose and 0.2 per cent NaCl. At 22 hours, a priming dose of 150 mgm./kgm. of PAH was given and an infusion of 2 cc./min. of 3.0 per cent PAH in 3 per cent glucose was instituted. This continued for 10 hours at which time a priming dose of 200 mgm./kgm. of PAH was given and the concentration of the infusion fluid was increased to 2 cc./min. of a 7.5 per cent PAH solution in 2 per cent glucose. Ten hours later the PAH administration was stopped and penicillin alone was continued for 12 hours. The total volume of fluid injected was 5640 cc. The weight of the animal increased from 12.9 kgm. to 15.5 kgm. during 54 hours.

Dog #102. Female. The preparation was similar to that described for preceding dogs. A priming dose of 1000 units of penicillin was given, followed by penicillin infusion at a rate of 15 units/min. for 16 hours, 30 units/min. for 10 hours and 60 units/min. for 10 hours, all concentrations in a saline-glucose solution being given at 1 cc./min. A salt-glucose infusion fluid, containing 0.45 per cent NaCl and 2.5 per cent glucose, was given at a rate of approximately 2 cc./min. throughout the experiment in order to maintain the urine flow from 1 to 3 cc./min. The total volume of fluid given during 36 hours was approximately 6.3 liters. The weight of 13.8 kgm. at the beginning of the experiment had increased to 19.2 kgm. at the completion of the infusion.

Dog #117. Female. This animal duplicated the experiment described for dog #102, with the exception of the concentration of the glucose-saline infusion fluid, which was changed to 0.225 per cent NaCl and 3.75 per cent glucose. The volume of fluid administered was approximately 5.9 liters. No weight was recorded for this animal at the completion of the experiment.

Dog #86. Female. A similar preparation was used for this dog as had been used with the preceding dogs. The penicillin was administered in an infusion fluid containing 0.225 per cent NaCl and 3.75 per cent glucose. For the intravenous drip solution a solution containing 0.1125 per cent NaCl and 4.375 per cent glucose was given at approximately 2 cc./min. During 36 hours this dog's weight increased 600 gm., from 13.6 kgm. to 14.2 kgm.

COMMENT. An examination of the gross and microscopic findings demonstrated that there were no lesions present in those animals given PAH plus penicillin, attributable to the administration of these drugs, which were not also present in those animals given saline-glucose or penicillin alone. Excepting those lesions due to intercurrent infections or parasitic infestations, which might

be expected to occur in any random selection of dogs, the following findings were common to most of the animals used in these subacute experiments.

Gross findings: There was marked subcutaneous edema. Pleural, pericardial and peritoneal fluids were increased in amount. The lungs, liver and kidneys usually showed moderate to marked congestion.

Microscopic findings: Kidney: Congestion was a constant finding. Parenchymatous degeneration of varying degrees of severity was observed, but was not associated with any particular treatment. Edema was an inconstant finding. Bladder: Edema and patchy mucosal ulceration associated with submucosal hemorrhage was observed irrespective of the drug used. Liver: Passive congestion and focal necrosis were always present in those animals treated with PAH plus penicillin and their controls receiving saline-glucose only; whereas only one of the three dogs given penicillin alone showed similar lesions. These changes seemed to be related to the volume and type of infusion fluid administered and to the rate of infusion rather than to any specific hepatotoxic action of PAH. Lungs: The most significant and common finding was vascular congestion. The myocardium, lymph nodes, spleen and skeletal muscle all displayed edema.

Generalized edema of varying degrees was a common finding in all dogs. This could be attributed to the constant intravenous infusion of large quantities of fluid. It was deemed necessary to resort to this measure in order to minimize the effects of variation in rate on the amount of material administered and the plasma concentration of penicillin and PAH. Even when the penicillin was administered by the constant rate infusion apparatus we felt it advisable to continue the drip infusion of PAH at 2 cc./min. so that control of plasma concentrations of PAH could be maintained.

The acute suppurative tracheitis, which was a constant finding, was due to the tracheal intubation which was carried out to assure an unobstructed air way and adequate aeration.

The frequent cystitis and ulceration of the bladder mucosa was traumatic in origin, due to the manual compression of the bladder against the indwelling catheter at the time of urine collection.

SUMMARY

No toxic manifestations were observed in white mice given oral doses of 2.0 gm./kgm of 20 per cent PAH every hour for four hours. Similarly, intraperitoneal administration of 0.2 gm/kgm. of sodium p-aminohippurate (PAH) (20 per cent), followed by oral doses of 2.0 gm./kgm. hourly for four hours, did not give rise to signs of toxicity.

The LD_{50} of PAH administered intravenously to white mice was determined to be 4.93 ± 0.8 gm/kgm. p-Acetylaminohippuric acid and hippuric acid, a structurally related body metabolite, had LD_{50} 's, determined in a similar manner, of 5.25 ± 0.5 gm/kgm and 1.15 ± 0.7 gm/kgm., respectively.

The administration of PAH to rabbits by constant intravenous infusion at rates from 2.2 gm/kgm hr. to 3.2 gm/kgm hr for periods of six hours resulted

in average 6 hour blood concentrations of 604 mgm./100 cc. of whole blood. The toxic manifestations and early deaths were not preeminently a function of blood concentrations, but were a function of the tonicity and the rate of infusion of the solution.

Dogs given constant intravenous infusions of PAH at rates designed to cause very rapid increases in plasma concentrations usually gave rise to toxic signs when such concentrations approximated 600 mgm./100 cc. The rapidity with which such high plasma concentrations were attained was deemed to be of greater importance than the plasma concentration *per se* in causing toxic manifestations. Histopathological examination of tissues taken from dogs that died following prolonged elevated plasma concentrations showed widespread passive congestion and edema, focal necrosis in the liver and toxic nephrosis.

Histopathological examinations of tissues from dogs given PAH and penicillin, or penicillin alone and from control animals given only the diluent (4) demonstrated no lesions that could be ascribed to the toxic action of PAH alone or to PAH in combination with penicillin.

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THE EFFECTS OF ANESTHESIA ON GLUCOSE TOLERANCE IN MAN¹

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The stimulating effect of ether on glycogenolysis in muscle and liver is well established. Other anesthetic agents, pentothal, cyclopropane and procaine are considered to have little effect on carbohydrate metabolism since a significant variation in the fasting blood sugar was not noted during or after their administration (1-2).

An investigation of carbohydrate metabolism during acute alcoholism in man revealed a markedly reduced blood sugar tolerance. To determine whether this phenomenon resulted from the specific action of alcohol or was due to anesthesia, the blood sugar tolerance in the same individuals was determined during other circumstances. Each subject was studied in the resting state without anesthesia and during surgical anesthesia with an intravenous barbiturate (pentothal), during inhalation anesthesia (cyclopropane) and during spinal anesthesia (procaine).

METHODS. Four volunteer normal adult males, who had been admitted in an acute alcoholic episode served as subjects. All had completely recovered, were clear mentally and had no evidence of nutritional deficiency or other physical illness. Each subject received all of the anesthetic agents used but not in any particular order. The order of testing was carefully altered in each series and between every test there was an interval of at least seven days. No preanesthetic medication was given at any time.

Cyclopropane was administered by the closed carbon dioxide absorption technique, an endotracheal airway was used and anesthesia maintained in the second plane of surgical anesthesia. Pentothal Sodium was given intravenously in 5 per cent solution in amounts required to prevent muscular activity. These patients received oxygen from an oropharyngeal catheter and were provided with a pharyngeal airway. Spinal anesthesia was induced and maintained with procaine at a sensory level approximating the fourth thoracic dermatome. In each instance the subject was maintained with the same degree or extent of anesthesia throughout the period of the experiment.

Each test was started with the subject post-prandial and at basal conditions. At a pre-determined time after surgical anesthesia was reached (45 to 60 minutes)

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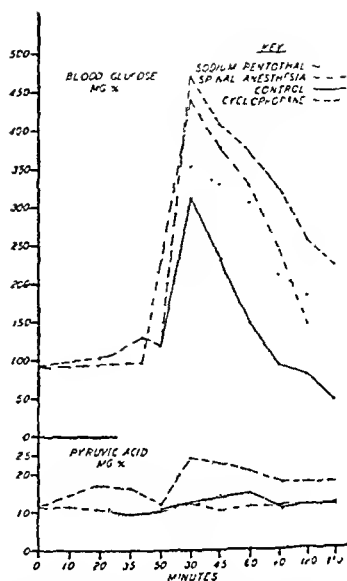
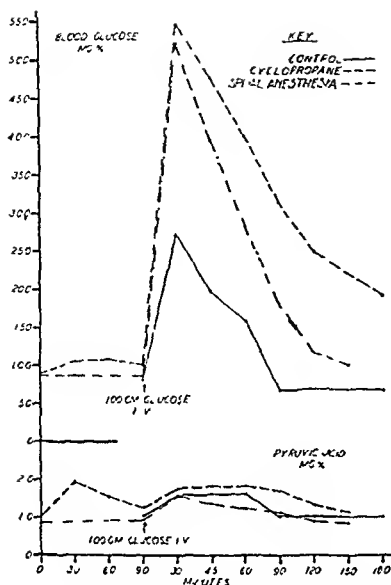


FIG. 1. GLUCOSE TOLERANCE CURVES AND PYRUVIC ACID LEVELS IN UNANESTHETIZED MAN AND AFTER SPINAL, CYCLOPROPANE AND PENTOTHAL ANESTHESIA

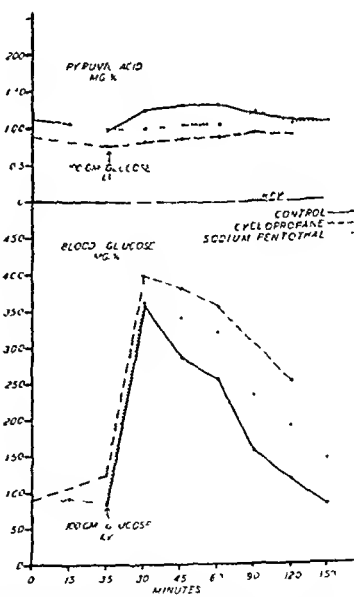
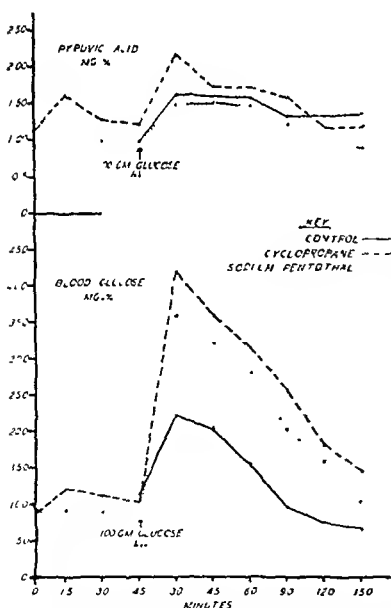


FIG. 2. GLUCOSE TOLERANCE CURVES AND PYRUVIC ACID LEVELS IN UNANESTHETIZED MAN AND AFTER CYCLOPROPANE AND PENTOTHAL ANESTHESIA

1.5 gms. of glucose (50% solution) per kilo body weight was injected intravenously. Blood sugar and blood pyruvate determinations were completed at regular intervals. The analytical methods were those used previously (3).

RESULTS. The plotted curve of blood glucose and blood pyruvates as determined are shown in figures 1 and 2. It will be noted that during Pentothal Sodium and spinal anesthesia there was no change in blood sugar but a temporary rise was present when cyclopropane was used. The blood sugar tolerance was decreased without exception with all three anesthetic agents studied. The impairment was most pronounced with cyclopropane.

A slight increase in blood pyruvic acid took place during cyclopropane anesthesia. The rise in blood pyruvate following glucose injection was essentially the same in the control experiment and during anesthesia with cyclopropane, pentothal and procaine.

CONCLUSIONS

Blood sugar tolerance was decreased during cyclopropane, pentothal sodium and procaine (spinal) anesthesia in each test in four humans. In contrast to anesthesia with pentothal and procaine, cyclopropane produces a slight elevation in blood sugar. This together with a rise in blood pyruvate might indicate that this anesthetic has an effect analogous to ether in stimulating glycogenolysis in liver and muscle. It should be noted particularly that cortical or brain stem depression alone cannot account for the decreased blood sugar tolerance since it was observed with spinal anesthesia at a high level. The same phenomenon was observed with alcohol anesthesia indicating that a general depression of carbohydrate metabolism takes place during anesthesia.

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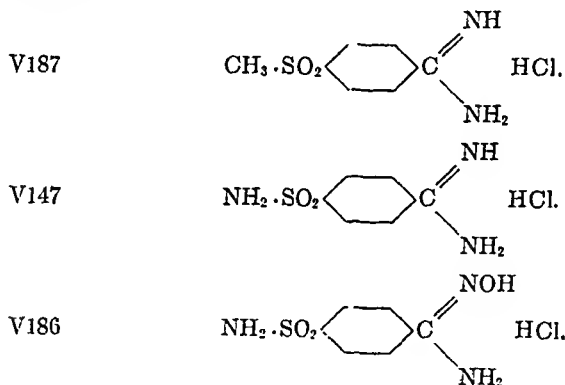
THE PHARMACOLOGICAL ACTION OF BENZAMIDINES AND BENZAMIDOXIMES

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In 1944 Andrewes, King, van den Ende and Walker (1) described the action of two new drugs V147 (p-sulphonamidobenzamidine hydrochloride) and V168 (p-sulphonamidobenzamidoxime hydrochloride) which prevented the development of pulmonary lesions in mice, after intranasal inoculation with rickettsiae of murine or epidemic typhus. A closely related compound V187 (p-methylsulphonylbenzamidine hydrochloride) was also shown by Evans, Fuller and Walker (2) to possess therapeutic activity against *Cl. welchii*, oedematiens and septicum in experimental animals.



This paper describes the pharmacological action of these three compounds.

Toxicity. The LD₅₀ for V187 in mice on intraperitoneal injection was found to be 1.2 g./kg. Of 30 mice receiving 1.5 g./kg. 28 died: of 30 receiving 1.0 g./kg. 9 died. This figure agrees well with that recorded by Evans, Fuller and Walker (2) for V187. These authors and Andrewes and his collaborators (1) have shown that the toxicity of the benzamidine V147 and the benzamidoxime V186 for mice is of the same order of magnitude.

The symptoms of poisoning by the two benzamidines, V147 and V187, were very similar. Within a few minutes of receiving a lethal dose intraperitoneally mice became distressed and the respiratory rate increased, though it decreased terminally: cyanosis and convulsions followed and the majority of such mice died within ten minutes. These symptoms are attributed to a profound fall of blood pressure, accompanied by respiratory failure and anoxial convulsions. In contrast the benzamidoxime V186 did not produce such dramatic results. The mice

became ill and hunched-up, the respiratory rate increased, but death was often delayed for several hours. This difference between the lethal symptoms of the benzamidines and the benzamidoximes is quite striking, and may be related to the difference between their vascular actions.

Unanaesthetized rabbits were also subjected to large single doses of V187 and V147 intravenously. There was little obvious disturbance until doses of 50 mg./kg. were reached, when the animals panted for a few minutes. With a dose of 200 mg./kg. (given intravenously in an 8% solution over 30 seconds) there was considerable distress for the next 10-15 minutes. Respiration became rapid and shallow, there was a rise in pulse rate, shivering and cleaning movements of the fore-paws over the nose. Though the rabbits looked dejected for the next two hours, all survived.

Heart. None of the three drugs has a direct action on the heart. Thus 5-10 mg. V187 or V147 injected into the fluid passing through the coronary vessels of the cat heart perfused with Ringer-Locke (Langendorff preparation) did not affect the force or rate of the contractions, nor was the coronary outflow altered. In one such preparation V187 was perfused in a concentration of 1 in 2,500 for 15 minutes without any change in the strength of the heart beat. Similarly 30 mg. V187 or V147 applied to the isolated auricle of a rabbit suspended in a 50 ml. bath (to give a final concentration of 1 in 1,700) had no effect. 40 mg. V147 injected into the isolated dog heart-lung preparation had no action on the left auricular pressure.

V186, however, is strongly acid in solution, and although it caused no cardiac irregularity in the intact animal, it did depress the Langendorff cat heart or isolated rabbit auricle owing to this acid reaction, since these preparations are suspended in Ringer-Locke. When the solution of V186 was buffered with Sodium Acetate, the depression previously observed in these isolated organs was no longer seen.

Circulation. Circulatory effects were observed in rabbits and cats; spinal or decerebrate preparations were used, or the animals were anaesthetized with chloralose, pentobarbitone or urethane. The two benzamidines, V187 and V147 behaved similarly and had a two-fold action; small doses caused a small rise of blood pressure, larger doses caused a profound fall (figs. 1 and 2). Thus 2.5-5.0 mg./kg. caused a rise of up to 15 mm. Hg.; 50 mg./kg. caused a fatal fall of pressure in spinal cats if repeated two or three times. Since both the rise and fall of blood pressure were seen equally well in the spinal animal, it is probable that they are both due to a direct peripheral action on the vessels. This view is supported by other observations: for instance in a cat anaesthetized with chloralose the intra-arterial injection of V187 (by a catheter passed down the left carotid artery to the aorta) caused a fall of blood pressure in half the dose required to produce the same effect by intravenous injection (fig. 2). This result is explained by the dilution of the material injected intravenously during its passage through the heart and lungs before it reaches the peripheral vessels. A direct action on vessels was also seen in the isolated hindlimb of the dog, perfused with defibrinated blood from a Dale-Schuster pump: in this preparation 20-40 mg. V187,

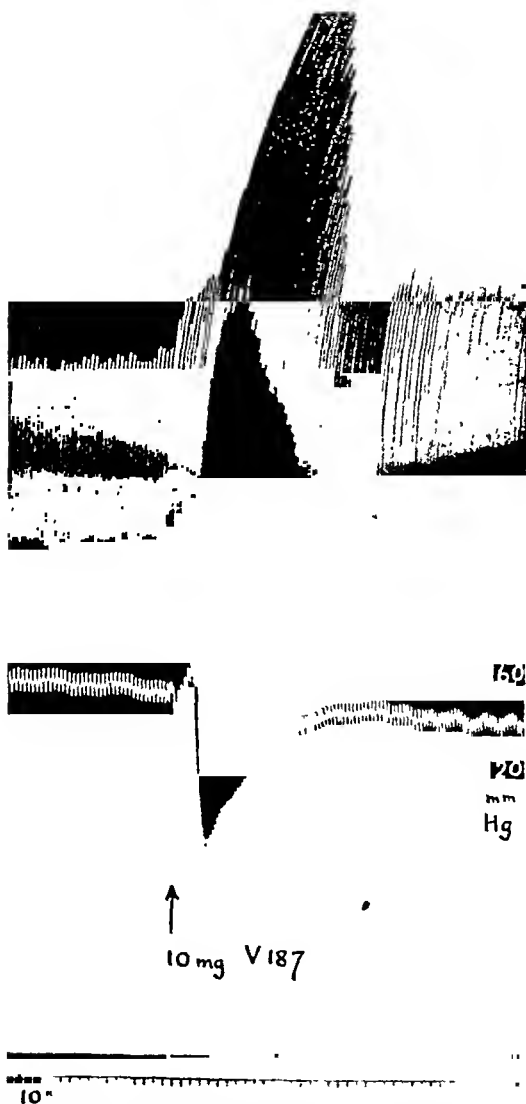


FIG. 1. CAT ANAESTHETIZED WITH SODIUM PENTOBARBITONE

Stimulation of respiration (above) and fall of blood pressure (below) on intravenous injection of 10 mg V187.

injected into the arterial cannula, caused a fall of arterial pressure accompanied by an increased outflow (fig. 3). In a similar preparation as little as 4 mg. V147 caused vasodilatation, particularly during an infusion of adrenaline, while still

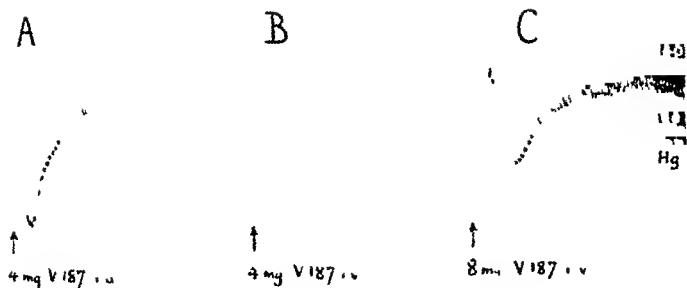


FIG. 2. CAT ANAESTHETIZED WITH CHLORALOSE

Injection of 4 mg V187 from a catheter passed down the carotid artery to the aorta (A) causes a fall of blood pressure. An equal dose injected by the jugular vein (B) has no such action, and it requires double the dose (C) to produce a similar fall by this route.

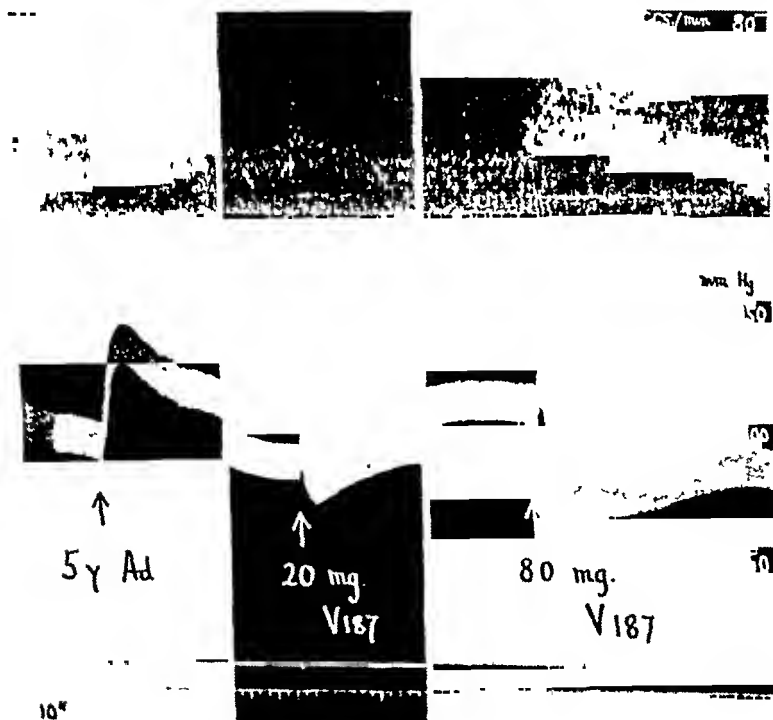


FIG. 3. DOG'S HINDLIMB PERFUSED WITH DEFFIBRINATED BLOOD FROM A DALE-SCHUSTER PUMP

Above: outflow. Below: pressure in the arterial cannula. 5 μ g adrenalin causes vasoconstriction. V187 causes vasodilatation when injected into the perfusion fluid, and increased outflow.

smaller doses caused a very small vasoconstriction. The action of these drugs on the circulation is therefore principally due to a direct effect on the peripheral vessels.

In contrast to the two benzamidines, the benzamidoxime V186 has far less effect on the circulation. From 5-50 mg./kg. V186 may cause no change in blood

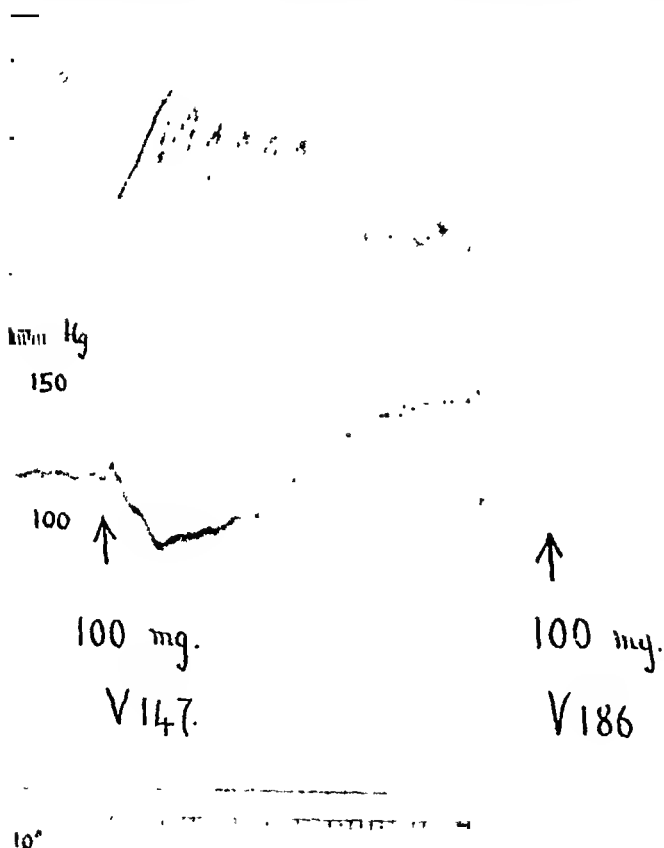


FIG. 4. SPINAL CAT

Record of spleen volume above, and of blood pressure below. 100 mg. V147 injected intravenously causes a dilatation of the spleen accompanied by a fall of blood pressure. 100 mg. V186 has virtually no action.

pressure (fig. 4) or a small rise. In other experiments from 15-90 mg./kg. has caused a transient fall of blood pressure (fig. 7). The vasodilator action of V186 has been seen in the perfused dog's hindlimb, but is less striking than that of V147.

Spleen. The depressor action of large doses of V147 and V187 is accompanied

by dilatation of the spleen (fig. 4). In contrast V186 in similar doses has no such action. This observation is in agreement with the effect of the three drugs on the blood pressure. The two benzamidines cause relaxation of the smooth muscle in the walls of the arterioles and in the spleen, while the corresponding benzamidoxime is much less active.

Other circulatory effects. Since calcium is known to affect certain of the pharmacological actions of guanidine, and since Wien (3) has shown that calcium reduces the fall of blood pressure caused by the intravenous injection of aromatic diamidines in the cat, it was of interest to see whether the same was true of V147 and V187. In a spinal cat (2.9 kg.) an injection of 100 mg. calcium chloride reduced but did not abolish the fall of blood pressure caused by 160 mg. V147 and 50 mg. V187.

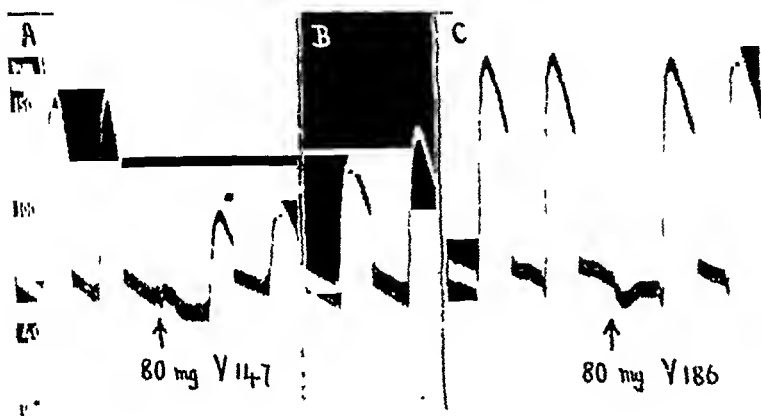


FIG 5 SPINAL CAT

Adrenaline in doses of 20 μ g was injected every 2-3 minutes. In A 80 mg V147 substantially reduces the pressor action of adrenaline. Between A and B there was an interval of six minutes, between B and C fifteen minutes. In C, 80 mg V186 does not modify the action of adrenaline.

Wien (3) also showed that the four aromatic diamidines which he investigated, and particularly stilbamidine, greatly reduced the pressor action of adrenaline. V147 and V187 have the same action in large doses (e.g. 40 mg./kg.). Much smaller doses have occasionally led to a slight increase in the pressor action of adrenaline in the spinal cat. On the other hand V186 does not reduce the pressor effects of adrenaline (fig. 5). It is tempting to correlate the considerable depressor action of the two benzamidines (V147 and V187) and their ability to relax the spleen with this property of antagonizing the pressor action of adrenaline, while the benzamidoxime V186 which is notably less active in these respects shows no such antagonism.

Capillary Permeability. The vessels of the frog's and rat's hindlimbs were perfused with Ringer-Locke solution containing 0.25% gelatine by the method de-

scribed by Hyman and Chambers (4). A single injection of 5 mg. V187 into the perfusion fluid, or perfusion with V187 in a concentration of 1:2,000, did not alter the rate of increase in weight, due to the slow onset of oedema. Hence V187 in large doses does not increase capillary permeability or lead to oedema formation.

Respiration. Respiration was recorded by a modification of Gaddum's apparatus (5) in cats anaesthetized with Sodium Pentobarbitone or decerebrate, and in rabbits anaesthetized with urethane. All three drugs cause an increase in the rate and depth of respiration. Fig. 1 illustrates a typical response to the injection of V187 in a cat under barbiturate anaesthesia. Usually this stimulation of respiration was associated with the fall of blood pressure, and might therefore be attributed to a reflex from the carotid sinus. In some decerebrate cats, however, injection of 2.5-5 mg./kg. V147 or V187 caused a rise of blood pressure of 10 mm. Hg. accompanied by a prolonged stimulation of respiration. A decerebrate cat was therefore prepared in which both carotids were ligated below the carotid sinus, and both vagi cut, in order to exclude peripheral vaso-motor reflexes. Injection of 20-40 mg./kg. V147, V186, or V187 caused a considerable increase in the depth of respiration, with no change in rate and in this instance very little alteration in the blood pressure. Quite apart from the vascular reflexes therefore, these drugs have a direct action on the respiratory centre. Large doses of V147 and V187 lead to a depression of respiration after the initial stimulation and this depression is also believed to be due to a direct action on the medulla oblongata, since it has been observed in some preparations in which the blood pressure rapidly recovered to a normal level. V186 did not cause depression of the respiration when injected in single doses up to 100 mg./kg., but when the drug was slowly infused into cats under Sodium Pentobarbitone anaesthesia, the initial stimulation was soon followed by a depression. The difference between V186 and the two benzamidines was therefore only one of degree; V186 was less active. There is no doubt that the depression of respiration caused by large doses of these drugs is the cause of death when a lethal dose is given intravenously: this much is made clear by experiments described below in which the drugs were infused slowly into anaesthetized animals.

The effects of V147, V186 and V187 upon respiration suggested that they might stimulate other medullary centres. While there is no unequivocal evidence that they affect the vasomotor centre, they cause vomiting in the pigeon: in this bird it is believed that the vomiting centre is located in close proximity to the vasomotor and respiratory centres. Pigeons weighing 290-360 grammes were used and the drug was injected slowly into the wing vein. Table 1 shows that whereas V187 causes vomiting in 50% of pigeons in a dose of ca. 50-100 mg./kg., and V147 in a dose of ca., 100-200 mg./kg., V186 does not cause vomiting at all up to the lethal dose.

Since these drugs were able to stimulate respiration, it seemed worth while testing them as analeptics. Mice received a dose of Sodium Pentobarbitone intraperitoneally sufficient to kill 50% and to keep the survivors narcotized for some hours. Simultaneous administration of V186 (chosen because it had less depressant action on the blood pressure) in a dose up to half the LD50 caused a

considerable stimulation of respiration compared with the control group, but did not shorten the duration of narcosis or reduce the fatality rate.

Intestinal movement. The action of these drugs on intestinal movement was studied both in vivo and on the isolated duodenum of the rabbit, suspended in oxygenated Ringer-Locke at 37°C. In the latter preparation both V147 and V187 caused a diminution in intestinal movements in a concentration of 1 in 50,000 or more. V147 was somewhat more powerful than V187 (fig. 6). V186

TABLE 1

DOSE INJECTED MGS	NUMBER OF PIGEONS IN WHICH VOMITING OCCURRED		
	V147	V186	V187
15	0/2	0/2	3/10
30	3/10	0/4	6/10
60	9/10	0/2	
120		0/2	
240		0/1 (died later)	

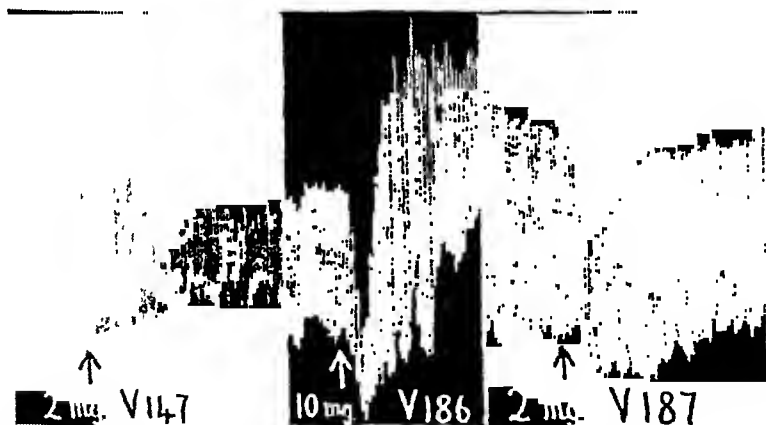


FIG. 6. ISOLATED RABBIT DUODENUM SUSPENDED IN OXYGENATED RINGER-LOCKE AT 38°C.
Capacity of bath 50 cc V186 is five times less active than V147 or V187

was 5-10 times less active than the two benzamidines, and in a concentration of 1:5,000 caused a transient inhibition of intestinal movements followed by a rise of tone, even when the solution was buffered.

By means of a balloon introduced into the duodenum, the changes of intestinal pressure were studied in spinal cats, or in cats under chloralose or sodium pentobarbitone anaesthesia. Small doses of V187, insufficient to cause a change of blood pressure, had a variable action, while small doses of V147 always caused an inhibition of peristalsis. Large doses of both V147 and V187 caused an initial stimulation of peristalsis with a rise of tone which outlasted the blood pressure

changes (fig. 7) and this was often followed by a prolonged inhibition. V186 had no action in comparable doses. These results are taken to indicate that the direct action of V147 and V187 on the intestines *in vivo* is depressant, as it is on the isolated duodenum of the rabbit; with larger doses this depression may be masked owing to the vascular changes. The action of these drugs on the intestines is, however, so mild that they are very unlikely to cause undesirable symptoms when used on man.

Effects of slow infusion. V147 and V187 injected intravenously in single doses of 5 mg./kg. or more caused a considerable fall of blood pressure and stimulation of respiration. It was therefore important to discover the threshold rate of absorption at which these effects might be expected to occur after, for instance, oral

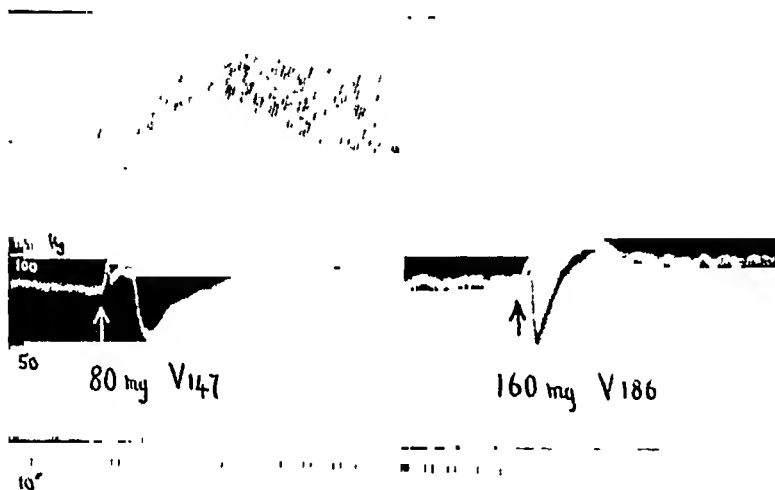


FIG. 7. SPINAL CAT (2.0 Kg.)

Above: record of pressure changes from a balloon in the duodenum. Below: blood pressure. V147 stimulates intestinal peristalsis and causes a rise of tone in large doses; V186 is inactive.

administration or local application to a wound. For this purpose the drugs were infused slowly into cats under barbiturate anaesthesia for periods of 15 minutes at a time. The results are summarized in table 2. Under these conditions V147 was less toxic than V187, and V186 was relatively innocuous.

By means of this method of slow infusion the lethal dose was estimated in cats anaesthetized with sodium pentobarbitone (25 mg./kg.). The drugs were infused intravenously at a uniform rate of 26 mg. per minute until the blood pressure fell to zero after respiratory failure. In five cats treated in this way the lethal dose for V147 was 0.31 ± 0.11 gm./kg. and for V187 was 0.28 ± 0.07 gm./kg.; in three cats the corresponding figure for V186 was 2.36 ± 0.78 gm./kg. Thus the benzamidoxine V186 was eight times less toxic than the corresponding benzamidine. It should, however, be observed that this method only provides a

measure of the acute toxicity of the drug which is being studied; the duration of the experiment is very short, and does not allow time for the development of toxic effects on, for instance, the liver or kidneys if in addition the drug causes respiratory or circulatory failure.

These results are encouraging in one respect, for they show that a very rapid rate of absorption of these drugs must be attained before any undesirable symptoms develop. It was foreseen that V187 in particular might be applied locally to wounds, and one further experiment on this drug was carried out. A 3 kg. cat was anaesthetized with sodium pentobarbitone and 45 minutes later, following a clean skin incision, a series of deep cruciate lacerations was made into the quadriceps muscles of both sides. No attempt was made to check haemorrhage other than packing the wounds with a total of 600 mg. powdered V187, and immediate suture of the skin edges. The disturbance caused by this operation subsided within a few minutes; there was no change in the blood pressure, respiration or duodenal movements (as measured by an intestinal balloon) during the next two hours. This dose of V187 would correspond to 12 gm. in a 60 kg. man.

Kidneys. Two types of acute experiment were undertaken on V187 only. In the first a 2 kg. rabbit was anaesthetized with urethane and the flow of urine

TABLE 2

	RATE OF INFUSION IN MG./KG./MIN. INTO CATS ANAESTHETIZED WITH SODIUM PENTOBARBITONE		
	V147	V186	V187
No effect up to	4		2
Fall of blood pressure	5-6	None up to 20	3.5-4.0
Stimulation of respiration	5-6	8-15	3.5-4.0
Depression of respiration	12-15	None up to 20	5-10

recorded from a cannula tied into the bladder. A diuresis was produced by a saline infusion and by injection of theophylline sodium acetate. V187 in doses of 10-30 mg. intravenously had no effect on the diuresis, except for the transient cessation of secretion due to the fall of blood pressure.

In another experiment two groups each of 4 rats, were given 10 cc. of water/200 g. by stomach tube and 0.012 unit posterior pituitary extract/200 g. subcutaneously. One group in addition was given 20 mg. V187/200 g. intraperitoneally. A week later the experiment was repeated, reversing the control and experimental groups. V187 did not modify the antidiuretic action of the posterior pituitary hormone.

In these acute experiments, therefore, there is no evidence that V187 has any immediate effect on kidney function.

Blood Sugar. The blood sugar was estimated by Somogyi's (6) modification of the Hagedorn and Jensen (7) method. Eleven rabbits starved overnight, were given V187 by the ear vein on a number of occasions. With the lower doses of 7.5-15.0 mg./kg. there was an occasional small rise of blood sugar from the resting level of 20-40 mg.%. With higher doses of 0.05-0.2 g./kg. there was either no change during the ensuing 24 hours, or a small fall. It was concluded that V187

had no material effect on the blood sugar in the doses used, which approach the lethal dose (1.2 g./kg. for mice) and certainly cause a profound vascular disturbance.

Salivary Secretion. The secretion of saliva was recorded by the outflow from a cannula inserted into the salivary duct of a cat anaesthetized with chloralose. A steady flow of saliva was produced by a slow infusion of pilocarpine 0.005 per cent with adrenaline 1:100,000. In two such preparations V187 in doses up to 20 mg. intravenously had no effect on the secretion of saliva unless there was a profound fall of blood pressure, when the flow was reduced, but rapidly returned to normal as the blood pressure rose again.

Reflex activity of the spinal cord. A flexor reflex was elicited in the spinal cat by stimulation of the posterior tibial nerve, while the contractions of tibialis anterior were recorded. Injection of up to 20 mg. V187 intravenously had no effect on the tension produced in the muscle when the afferent nerve received condenser discharges at the rate of one every 10 seconds. V187 under these conditions does not interfere with a simple spinal reflex, the transmission of the impulse from nerve to muscle, nor with muscular contraction.

Effects of chronic administration. Rats. A series of rats received the three drugs mixed with their diet over a period of 12-14 days. Two doses were used, 0.07 and 0.20 g./kg./day. On the lower dose the rats continued to gain weight as well as the control group: the results obtained with the higher dose are summarized in table 3.

These figures show that both V147 and V187 significantly reduce the growth rate of young rats in this dose. Yet the total amount of solid food consumed during this period did not differ between the control and experimental groups. The taste of the drug had not therefore interfered with the appetite of the rats. At the end of the experiment the rats were killed and their thyroids dissected out and weighed; there was no significant difference in the thyroid weights of the control and experimental groups and consequently these drugs do not cause thyroid hypertrophy under conditions in which thiourea may be expected to cause a significant increase in thyroid weight.

In a second type of experiment the blood urea was estimated from samples obtained by heart puncture, and the white cell count and haemoglobin (Lovibond comparator method) recorded before and after feeding 0.2 g./kg./day V187 for 14 days. In a group of 8 rats there was no change of blood urea, white count or haemoglobin either compared with previous readings or with a control group of rats fed on the diet alone.

Rabbits. A more stringent test of the chronic oral toxicity of V187 was undertaken on rabbits. Two rabbits were fed a dose of 0.07 g./kg./day, receiving this dissolved in 30 c.c. of water as a single dose by stomach tube every morning, for 14 days. There was no change in weight, in blood urea, white count or haemoglobin (using both the Lovibond comparator and Haldane haemoglobinometer).

Four other rabbits received a higher dose of 0.2 g./kg./day for 14 days by the same route. They continued to gain weight, and there was no material change in haemoglobin, white count or blood urea. Their urea clearance was unim-

paired, and there was neither albumen nor casts in the urine. Liver function before and after the experiment (for 2 months afterwards in the case of 2 rabbits) was assessed by the sucrose tolerance and hippuric acid tests (after administration of 4 g. sucrose and 0.6 g. sodium benzoate respectively by stomach tube). The blood laevulose was estimated by the diphenylamine method. As judged by these tests there was no impairment of hepatic function; the resting blood sugar was unaltered, the van den Bergh negative and there was no rise in plasma bilirubin. Since the question of intestinal irritation arose after such large oral doses of V187, the faeces of two of the animals were submitted to the benzidine test; no occult blood was detected.

In rabbits, therefore, V187 is well tolerated in large doses by mouth, and there is no evidence of kidney or liver damage as judged by these tests.

The Properties of Benzamidines and Benzamidoximes. The acute experiments described in the earlier part of this paper show that V186, which is a benzamidoxime, is much less active pharmacologically than the corresponding benzamidine V147. On the smooth muscle of the walls of arterioles, of the spleen and of the intestine this difference is particularly striking; it is also seen in the centres of the medulla oblongata, and in the growth rate of young rats. Wien (3) showed that four aromatic diamidines had a depressor action on the circulation

TABLE 3

Increase in weight in groups of 6 rats receiving 0.2 g./kg./day for 12 days

CONTROL	V147	V186	V187
25.5% \pm 3.0	14.7% \pm 7.9	20.4% \pm 6.4	11.1% \pm 4.4

even more powerful than V147 and V187 and also due to peripheral vasodilatation: this has been confirmed for stilbamidine, propamidine and pentamidine in the cat, and for pentamidine in the dog. The results obtained with V186 suggested that the substitution of amidoxime for amidine groups in these aromatic diamidines might reduce their depressor activity, and this was of greater interest because clinical experience of intravenous administration of these drugs in the treatment of leishmaniasis and trypanosomiasis in man had shown that the fall of blood pressure was sometimes followed by syncope and temporary collapse. For instance Lourie (8) records severe and immediate reactions after injection of more than 1-2.2 mg./kg. stilbamidine. Fig. 8 shows the comparative freedom of Stilbamidoxime (stilbene 4:4'-diamidoxime) from the depressant action of Stilbamidine on the cardio-vascular system, and from its stimulant action on the respiration. This suggests that substances containing the benzamidoxime group merit more attention so far as their therapeutic possibilities are concerned than they have yet received (Lamb and White, 9).

Discussion. In 1926 Alles (10) found that alkyl derivatives of guanidine caused a small fall of blood pressure in the rabbit under urethane anaesthesia, followed by a prolonged rise. This rise of blood pressure was of considerable interest at the time, as it was erroneously supposed to be connected with essential

hypertension in man. In 1917 Sinalnieoff and Bovshik (11) had concluded that the pressor effect of asym. dimethylguanidine was due to peripheral vasoconstriction, since it occurred after destruction of the spinal cord and section of the vagi; Goldblatt and Karsner (12) have confirmed this conclusion by plethysmographic experiments on dogs. The evidence that other alkyl guanidine derivatives cause a rise of blood pressure by a peripheral rather than a central action is not so good, but I have observed that methylguanidine will cause vasoconstriction in the dog's hindlimb perfused with defibrinated blood. Lewis and Koessler (13) found that

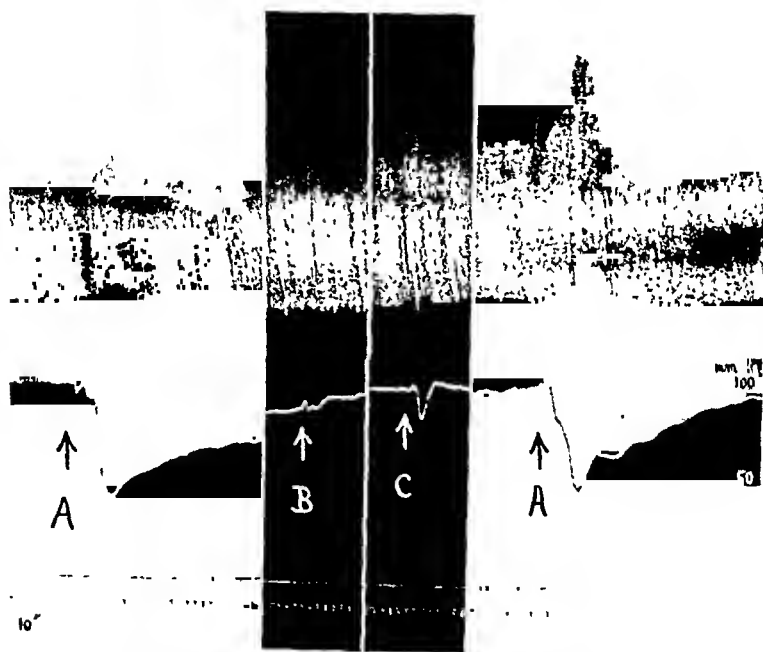


FIG. 8. Cat (2.2 Kg) ANAESTHETIZED WITH SODIUM PENTOBARBITONE.

Above: respiration. Below: blood pressure. Injection of 8 mg. stilbamidine isethionate at A causes a fall of blood pressure and stimulation of respiration. Stilbamidine in a dose of 16 mg. at B, and 32 mg. at C is relatively inactive.

guanidine, methyl- and dimethyl-guanidine caused contraction of isolated arterial strips, but the concentrations used were very high (1:100) compared with adrenalin (1:500,000).

Alles (10) also showed that two arylguanidines in smaller doses than the alkyl derivatives caused only a fall of blood pressure. While symmetrical diphenyl-guanidine caused an irregular record suggesting cardiac depression, triphenyl-guanidine caused no irregularity but a simple fall of pressure. Kuroda (14) investigated five other aromatic guanidine derivatives, all of which lowered the rabbit's blood pressure, an effect attributed to peripheral vasodilatation. Large

doses of these compounds depressed the rabbit's heart; in the perfused rabbit's ear (which does not show vasodilatation particularly well), while dilute solutions caused vasodilatation, concentrated solutions caused vasoconstriction. Junkmann (15) showed that decamethylene diguanidine (synthalin) also caused a fall of blood pressure in the rabbit under urethane anaesthesia; this was due to peripheral vasodilatation and not to an action on the heart. But in the perfused rabbit ear a low concentration caused vasodilatation, higher concentrations vasoconstriction; and in similar high concentrations synthalin caused toxic effects on the frog heart. These observations are very similar to Kuroda's. The introduction of an aromatic group therefore appears to increase the action of guanidine upon the cardio-vascular system; and the presence of two guanidine groups in the same molecule separated by an alkyl chain (as in synthalin) has the same effect. The pressor action of guanidine itself in the intact animal is reversed: this is due to peripheral vasodilatation, in some instances associated with a toxic effect on the heart. This generalization is, of course, dependent on the composition of the rest of the molecule, for the introduction of a carboxyl group as in arginine will completely abolish the circulatory effect (Thompson 16, de Silva 17).

Benzamidine derivatives also cause a fall of blood pressure, and the available evidence shows that this is due primarily to peripheral vasodilatation. Goodwin and Marshall (18) studied a large series of benzamidines and aromatic derivatives of acetamidine, which were found to cause a fall of blood pressure in a dose comparable to that in which V147 and V187 have a depressor action. The aromatic diamidines are about 20 times as active as the monobenzamidines, molecule for molecule, in causing vasodilatation; it is interesting to compare this increased toxicity with that of synthalin.

Smirk and his colleagues (19, 20) have shown that a large number of iso-thioureas having the general formula $R.S.C \begin{smallmatrix} \diagup NH \\ \diagdown NH_2 \end{smallmatrix}$ (when R is an alkyl group) cause

a rise of blood pressure due to peripheral vasoconstriction in experimental animals. S-methyl iso-thiourea sulphate had such a powerful pressor action that it

has even been advocated for clinical use. If $-C \begin{smallmatrix} \diagup NH \\ \diagdown NH_2 \end{smallmatrix}$ is the active group in

guanidines and amidines, the thioureas might be expected to conform to the principles enunciated above. Fastier and Smirk (18) state that whereas S-methyl, S-ethyl and S-iso-propyl iso-thiourea cause large rises of blood pressure in cats and dogs, with the S-n-butyl and S-tert.-amyl derivatives more variable results were obtained. While temporary rises were sometimes given by initial doses of S-n-butyl iso-thiourea, later doses produced only falls of blood pressure. This suggests that lengthening the chain beyond 3 or 4 carbon atoms may reverse the pressor action. The effect of introducing a benzene ring on the vascular action of thioureas is not recorded, substances of this type are more toxic but not very soluble (Hanzlik and Irvine, 21; Richter and Clisby, 22).

The action of guanidines, amidines and thioureas on the respiration is complicated by their vascular effects, and not all workers have differentiated between

the reflex stimulation following a fall of blood pressure, and a direct action upon the medulla. However guanidine in doses which had a pressor action was shown by Alles (10) to stimulate respiration, followed by depression. This is very like the action of the monobenzamidines V147 and V187: Fastier and Smirk (20) observed a similar stimulation accompanied by a rise of blood pressure with various thioureas.

Guanidine derivatives in general have a toxic action on the liver. This has been abundantly proved for guanidine itself and for decamethylene diguanidine. The investigations of Bischoff and Long (23) and of Broom (24) show that many other guanidines, monoamidines, alkylene diamidines and benzamidines cause liver and kidney damage in animals. These changes were seen after single injections of the drug only as the LD50 was approached. The aromatic diamidines have also been shown to cause liver damage both in therapeutic doses (Daubney and Hudson, 25, Fulton and Yorke, 26) where clinical work suggests that there may be a deceptive delay before the onset of toxic symptoms, and in experimental animals (Wien, Frecman and Scotcher, 27, Allen, Burgess and Cameron, 28). The experiments of Wien and his colleagues, like those of Broom showed that the blood sugar was affected only as the lethal dose was approached. The action of these drugs on the blood sugar does not necessarily depend on liver damage alone, as Bodo and Marks (29) showed for synthalin. Both hyperglycaemia and hypoglycaemia may be produced according to the dose and chemical structure. V187 in doses of 0.2 g./kg. had no appreciable effect on the blood sugar in rabbits, and did not cause liver or kidney damage in rats or rabbits after a fortnight's oral administration. This dose is considerably less than the LD50 (1.2 g./kg. for mice), for these experiments were designed to indicate the safety range of this drug for use in human beings. But in view of the formidable evidence of liver damage caused by closely related compounds, still larger amounts of V147 or V187 may be expected to have a similar effect.

Various thiourea derivatives are known to cause thyroid hyperplasia on prolonged administration (MacKenzie and MacKenzie 30, Astwood 31). Both authors found that guanidine salts were inactive in this respect, so that it is not surprising to find that the two benzamidines V147 and V187 are also inactive. Conversely (though of more theoretical than practical importance), it would be interesting to know more of the action of thiourea and S-methyl isothiurea on the liver. In the doses used for the treatment of thyrotoxicosis, thiourea has not been shown to cause liver damage; but this effect was only demonstrated in guanidine and amidine derivatives as the lethal dose was approached.

SUMMARY

1. When single doses of the benzamidines V147 or V187 are given intravenously to experimental animals they produce:
 - a. A rise of blood pressure if the dose is small
 - b. A fall of blood pressure due to peripheral vasodilatation if the dose is larger than 5 mg./kg.

- c. A stimulation of respiration, partly due to the fall of blood pressure and partly due to a central action.
- d. Vomiting when given to pigeons.
- e. Death due to respiratory failure after excessive doses.
2. V186, a benzamidoxime, resembles V147 and V187 in having no direct action on the heart. It is much less active pharmacologically and this difference has been demonstrated on the smooth muscle of the walls of arterioles, of the spleen and of the intestine; in the respiratory and vomiting centres of the medulla, and in the growth rate of young rats. This benzamidoxime is therefore less toxic in acute experiments than the analogous benzamidine.
3. V187 has no appreciable effect in single injections on the blood sugar, salivary secretion, spinal reflexes or capillary permeability. It may be infused slowly into the anaesthetized cat at a rate of up to 5 mg./minute without alteration of blood pressure, respiration or intestinal movement: this rate of absorption is unlikely to be reached after local application of the drug to wounds.
4. V187 has been fed to rabbits and rats in a dose of 0.2 g./kg./day for two weeks without affecting the blood count, liver or kidney function.
5. The relation between the structure of guanidine, amidine and thiourea derivatives and their pharmacological actions is discussed.

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A METHOD FOR THE ASSAY OF ATROPINE SUBSTITUTES ON THE SALIVARY SECRETION

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In 1904 Cushny (1) published his first paper on optical isomers, using atropine and the hyoscyamines, and in the course of this work described a method for the comparison of the activity of those substances on the salivary secretion. This method, which he used in all subsequent work, measured the antagonism of the belladonna alkaloids to pilocarpine when injected into an unanaesthetized dog with a salivary fistula; the saliva was collected on cotton-wool which was weighed at intervals of 5 minutes. The activity of the unknown substance relative to that of atropine was estimated in a very limited number of dogs, and as Cushny (2) showed, the reaction of the individual dog to the weaker tropines is liable to unpredictable changes over a period of months.

Issekutz (3) anaesthetized rabbits with urethane, and laid them on their side so that the saliva was not swallowed, but flowed into a small dish which was weighed at 10 min. intervals. Like Cushny he measured the inhibition of a pilocarpine secretion by atropine derivatives, and his method is equally slow.

Nyman (4) used a very ingenious method of measuring the salivary flow from one parotid gland in human beings. Pilocarpine was injected subcutaneously or infused intravenously to cause a secretion of saliva. Since the belladonna alkaloids used were slowly absorbed, they were injected subcutaneously half-an-hour before a subcutaneous injection of pilocarpine. There was a linear relationship between the dose of the belladonna alkaloid and the logarithm of the total quantity of saliva secreted during 60 minutes after the injection of pilocarpine, and from this relationship the relative potency of the alkaloids used was calculated. When pilocarpine was infused intravenously to give a steady secretion of saliva, the results were less satisfactory. A fast flow of saliva, which was more readily inhibited by atropine, could not be used because of other undesirable effects of pilocarpine. The error of the estimation with a medium salivary flow was large, as the end point of the effect of the belladonna alkaloid was difficult to determine.

None of these methods is suitable for the assay of large numbers of atropine substitutes on the salivary flow, because they are so time-consuming. This is due either to the use of large doses of atropine (Cushny (1, 2) and Issekutz (3)), or to a slow salivary flow. As Nyman observed, the inhibitory action of belladonna alkaloids is far greater when the salivary flow is rapid, and the essential modification we have introduced is that the sialogogue used is infused intravenously to give as fast a flow as possible, consistent with a constant rate of secretion. Under these conditions the duration of action of atropine is far shorter and many assays may therefore be made on the same animal.

¹ During the tenure of a personal grant from the Medical Research Council.

METHOD. Cats are used, anaesthetized with pentobarbitone or chloralose. Both anaesthetics give equally satisfactory results. A cannula is tied into Wharton's duct and attached to a bottle containing tap water. The tap water, displaced by saliva, passes out of the bottle through the tube which actuates a drop timer (Gaddum and Kwiatkowski (5)). As each drop forms at the mouth of the tube it makes a contact which brings an electromagnet into action to record a vertical line on a drum. When the drop falls the contact is broken, and the vertical line is interrupted; the height of the line is thus proportional to the interval between one drop and the next. Records such as those seen in Fig. 1. are obtained. A steady flow of saliva is produced in the cat by an intravenous infusion at uniform rate of a 0.005% solution of pilocarpine or a 0.004% solution of carbaminoylcholine in Ringer-Locke. Since both these substances lower the blood pressure, adrenaline hydrochloride, 0.002%, is also included in the infusion. Adrenaline has been shown by Stavrakys (6) to increase the salivary flow produced by eserine, pilocarpine and mecholyl. It has been found an advantage to use carbaminoylcholine rather than pilocarpine nitrate. The effect of carbaminoylcholine on the blood pressure is promptly antagonised by atropine, whereas that of pilocarpine is not. As a result it is possible to compare atropine substitutes with atropine not only by their action on the salivary flow, but simultaneously by their action upon the blood pressure. The difference in the effect of carbaminoylcholine and of pilocarpine on the blood pressure is probably that the effect of choline esters is on the arteries whereas, as was shown by Burn (6) that of pilocarpine is, like that of histamine, on the capillaries.

The apparatus used for infusion is of some importance, for the rate of infusion must be very constant. Some of the earlier experiments were carried out using a 20 cc. syringe, the piston of which was slowly driven in by a worm-gear attached to a small electric motor. This was not very satisfactory, as the temporary cessation of the infusion while the syringe was refilled interfered with the assay. A device of larger capacity was necessary, which would deliver a constant volume per minute in spite of small fluctuations of venous pressure, and at a very slow rate of infusion. We are indebted to Dr. E. H. J. Schuster for the loan of an apparatus which fulfils these requirements. In principle this consists of two cylinders, one of which fills while the other empties; the movement of the pistons is controlled by a screw, which is intermittently rotated by a friction ratchet with a variable throw, driven by a small electric motor.

The rate of infusion of carbaminoylcholine is usually about 0.5 cc. per min., but it requires adjustment for each animal until optimal salivary flow is obtained. This is such that the intravenous injection of atropine sulphate in a dose of ca. 1-5 μ g. at intervals of 5 minutes no longer causes a gradual decrease in the rate of secretion over a long period. This optimal condition is only reached after some trouble; in practice it is best to run the infusion with intermittent injections of atropine for half-an-hour, and then to stop it for 45 minutes. At the end of this time results are more constant, the resting blood pressure steadier, and small doses of atropine can be injected every 3-5 minutes, causing a transient rise of blood pressure and decrease of salivary secretion, without cumulative action.

Fig. 1. shows the graded response to three different doses of atropine sulphate. It will be seen that, in order to obtain short lasting effects without cumulative action the range of dosage is limited. The period in which similar effects can be obtained is also limited, due to changes in the condition of the cat or the rate of infusion. Thus a given dose of atropine may cause a different degree of inhibition of salivary flow at various stages of the experiment. The assay of inhibiting substances was therefore carried out in the following way. A dose of atropine was chosen which gave neither too small nor too big an effect; (from the experiment shown in fig. 1 the middle dose, i.e. 3 μ g. would be selected). This dose was injected repeatedly until a uniform response was obtained.

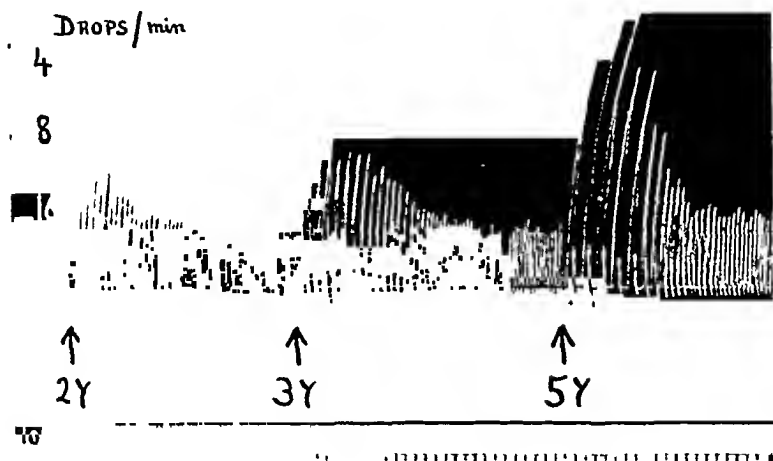


FIG 1 CAT

Pentobarbitone Slow intravenous infusion of carbammyloxycholine 0.004% and adrenaline 0.002%. Record of salivary flow. The inhibitory effect of three different doses of atropine sulphate is shown

The activity of the unknown substance was assayed against this dose of atropine sulphate by recording the effect of a given dose of the new compound twice between two records of the effect of a given dose of atropine. This procedure was followed until either an exact match was achieved, or until doses had been discovered which were more effective and less effective than atropine. The relative potency was calculated throughout by measuring the maximum effect of an injection. The duration of action was not taken into account as it could not be determined accurately. In the same animal the relative activity established in this way was constant for a given substance for as long as the preparation survived (up to 10 hours), and as many as 10-15 different substances could be assayed during the day.

EXPERIMENTAL RESULTS Eight known substitutes for atropine have been

tested by the method described; for each substance not less than 5 cats were used. Table 1 shows good agreement between the results obtained by the assay on

TABLE 1
Potency relative to atropine sulphate = 100

	ON SALIVARY SECRETION			ON BLOOD PRESSURE	NUMBER OF	
	Lowest	Highest	Mean		Cats	Observations
Atropine methylnitrate (Eumydrine)	133	250	190 ± 21	170 ± 13	5	9
l-Hyoseyamine sulphate	150	200	180 ± 12	190 ± 7.2	6	10
l-Hyoseyamine methiodide	300	343	310 ± 5.6	300 ± 3.6	6	11
d-Hyoseyamine hydrobromide.	7.5	8.7	8.2 ± 0.2	7.4	5	8
Hyoscine hydrobromide	66	200	120 ± 22	110 ± 14	5	11
Hyoscine methiodide . .	200	320	260 ± 22	260 ± 28	5	7
Eucatropine hydrochloride	2.5	6	3.65 ± 0.6	2.5	5	12
Eucatropine methiodide .	25	82	50 ± 2.2	5.6	5	13

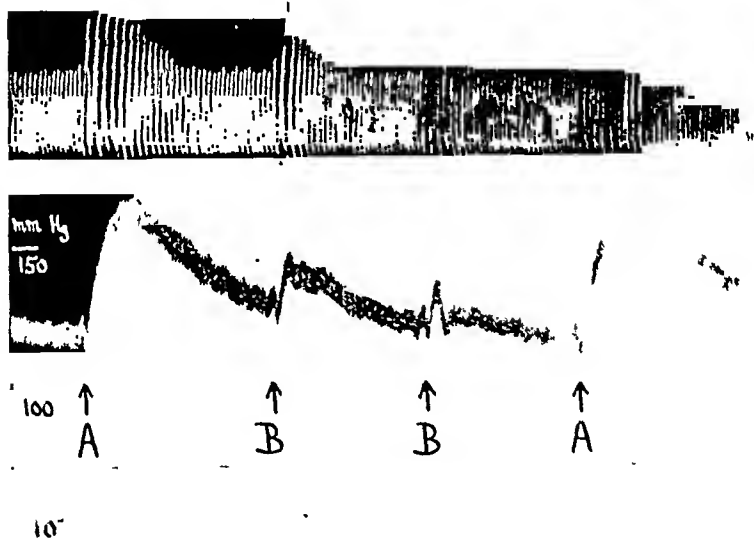


FIG. 2 CAT

Preparation as Fig. 1. Top record salivary flow; bottom blood pressure. At A intravenous injection of 2 μ g atropine sulphate, at B 3 μ g eucatropine methiodide.

salivary flow and that on the blood pressure, except for eucatropine methiodide. The range of variation of observations on the salivary flow is greater for hyoscine

hydrobromide and the eucatropine derivatives. Hyoscine hydrobromide has a much more prolonged action than atropine, while eucatropine has a much shorter effect. Such differences in duration of action make the assay more difficult as may be seen from fig. 2. In this assay 2 μ g. atropine sulphate caused a smaller inhibition than 3 μ g. eucatropine methiodide, but the eucatropine effect is very transient. From the same figure it is seen that eucatropine methiodide, though it has a powerful effect on the salivary flow does not affect the blood pressure appreciably. Of all the atropine substitutes tested only eucatropine methiodide showed this discrepancy.

In fig. 3 is shown a typical assay in which the effects on salivary flow and on the blood pressure are in agreement. The well known fact that l-hyoscyamine is twice as potent as atropine is demonstrated in the top tracing. In the bottom

TABLE 2

SUBSTANCE	CUSHNY (1, 2, 8, 9) DOG S C	NYMAN (4, 10) MAN S C	BULBRING AND DAWES CAT 1 A
Atropine sulphate	100	100	100
Atropine methylnitrate (Lunmydrine)	150	220	190
l-Hyoscyamine sulphate			180
l-Hyoscyamine hydrobromide	200	205	
l-Hyoscyamine methiodide			310
d-Hyoscyamine hydrobromide	5		8.2
l-Hyoscine hydrobromide	ca 200	300	120
l-Hyoscine methylnitrate		400	
l-Hyoscine methiodide			260
l-Hyoscine methylbromide		240	
Eucatropine hydrochloride		0.2	3.6
Eucatropine methiodide			50

tracing the corresponding quaternary salt, l-hyoscyamine methiodide, is seen to be stronger than the tertiary salt. This appears to be a general rule.

DISCUSSION. A new method has been described for the assay of atropine substitutes on the salivary secretion of cats stimulated by carbaminoylcholine.

Table 2 gives the results obtained by different authors using different methods for the comparison of the action of tropine derivatives on salivary secretion. There is good agreement for atropine methyl nitrate and for the tertiary bases l- and d-hyoscyamine. The results obtained with hyoscine and eucatropine derivatives do not show such good agreement and these are the compounds with which we obtained the greatest scatter during the assay, owing to the differences in the duration of action of these compounds and that of atropine. These discrepancies may be due to different methods of administration and to different rates of absorption and elimination. Both on the salivary gland and on the blood

pressure the quaternary compounds were found to be stronger than the tertiary ones. Nyman's results on man agree with this conclusion for atropine methyl

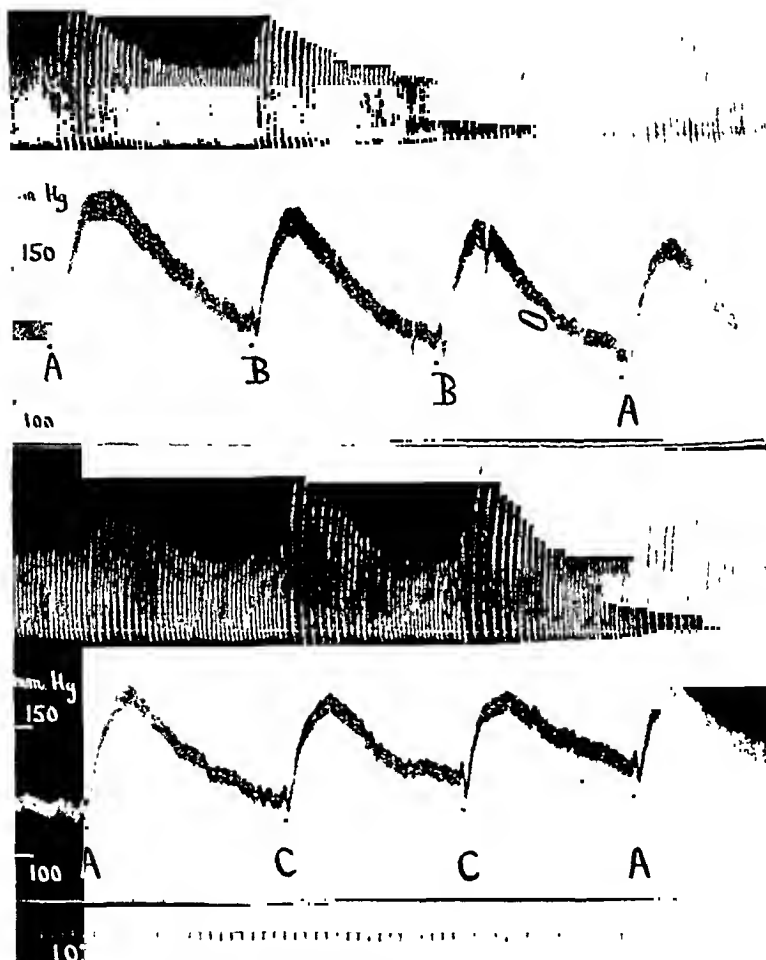


FIG 3 RECORDS AS FIG. 2

At A intravenous injection of $2\mu\text{g}$ atropine sulphate, at B $1\mu\text{g}$ 1-hyoscyamine sulphate, at C $0.7\mu\text{g}$ 1-hyoscyamine methiodide.

nitrate but not for hyoscyne methylbromide. We can explain this only by the fact that Nyman injected the drugs subcutaneously while we gave them intravenously.

SUMMARY

A method is described for the assay of atropine-like substances on the salivary secretion and the blood pressure of the cat during a slow intravenous infusion of carbaminoylcholine. The advantage of the method is that large numbers of compounds can be tested in a short time.

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COMPARISON OF THE PHARMACOLOGIC ACTION OF QUINIDINE AND DIHYDROQUINIDINE

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The action of quinidine in restoring normal rhythm to the fibrillating heart is well known and has been widely studied. Apparently, much less recognized is the fact that commercial quinidine sulfate, U.S.P., contains appreciable amounts of impurities in the form of other cinchona alkaloids. Dihydroquinidine is the chief contaminant, as first reported by Lewis et al. (1, 2), being present usually to the extent of about 20%. Lewis and co-workers made comparative tests of the action of pure preparations of these compounds on human beings with chronic auricular fibrillation. They concluded that dihydroquinidine was very slightly more powerful than quinidine. No further mention of this action of dihydroquinidine is found in the literature until 1937, when Van Dongen and Sanches (3) reported that pure quinidine appeared to be without effect in increasing resistance to fibrillation by electrical stimuli to cats' hearts. The action of commercial quinidine sulfate was attributed by them entirely to its dihydroquinidine content. Apparently these authors overlooked the earlier work of Lewis, since they made no reference to it. Weisman (4), who studied the depressor action of these drugs, mentioned in a footnote that hydroquinidine failed to restore normal cardiac rhythm to any of 8 patients with chronic auricular fibrillation.

The present World War has caused an imminent exhaustion of quinidine. Drs. R. B. Woodward and W. E. Doering, of Harvard and Columbia Universities, respectively, working with the Office of Production Research and Development of the War Production Board, developed methods useful in the manufacture of synthetic quinidine and dihydroquinidine. They invited our laboratory to participate in the pharmacologic investigation. In view of the favorable reports published in Europe and referred to above, dihydroquinidine was included in the present study. Samples of pure quinidine and dihydroquinidine were generously supplied by Drs. Woodward and Doering, while that of commercial quinidine sulfate, U.S.P., was obtained from our own stock. The melting points and specific rotations of the 3 products are as follows: 170.5–171.5°C., and $[\alpha]_D^{27} +269.6^\circ$ in 95% ethanol, for quinidine; 169.5–170.5°C., and $[\alpha]_D^{27} +213^\circ$ in 95% ethanol, for dihydroquinidine; and 212–214°C. with decomposition for commercial quinidine sulfate, U.S.P.

Pharmacologic Studies. 1. *Effect on Ventricular Fibrillation in Cats.* The action of dihydroquinidine and quinidine against ventricular fibrillation was studied by a procedure essentially the same as that of Lindner and Katz (5). This consisted of tetanic stimulation with a Harvard inductorium of the right ventricle near the A-V septum. Cats anesthetized with pentobarbital sodium were used, the thorax being opened and artificial respiration maintained. Blood

pressure was recorded from the carotid artery. Three dry cells were connected in series to the primary coil of the inductorium. The duration of each stimulus was kept constant, at approximately 1 second throughout all tests. Each strength of stimulus was tested 3 times before increasing. The threshold for permanent ventricular fibrillation was determined. Fibrillation was considered permanent after 60 seconds, following which normal rhythm was reestablished by counterstimulation of the ventricles for about 1 second with 110 volts A.C., according to Hooker and associates (6). After 20 to 30 minutes for recovery, the fibrillation threshold was again ascertained. These latter results proved to be much less consistent than the first threshold measurements. One series of cats was studied without any quinidine preparation to serve as controls. The other animals were given an intravenous injection of either pure quinidine, dihydroquinidine, or commercial quinidine sulfate just prior to measurement of the fibrillation threshold. Since these drugs have depressor action, the blood pressure was always allowed to return to normal before electrical stimulation was initiated.

The results of these tests of cardiac action are shown in table 1. Dihydroquinidine is obviously more potent than pure quinidine in raising the threshold current needed to produce permanent ventricular fibrillation in cats. A dose of 1 mg. per kg. intravenously appeared to be the threshold dose for dihydroquinidine, while with pure quinidine, the same effect was produced with about 3.5 mg. per kg. Dihydroquinidine is thus approximately 3.5 times as effective as pure quinidine in its action on the heart in this respect. Commercial quinidine sulfate, as would be expected, was intermediary, its threshold dose being about 2.0 mg. per kg. These results agree with those of Lewis et al. (1, 2) and Van Dongen and Sanches (3), except that the latter authors could demonstrate no cardiac action whatsoever for pure quinidine.

2. Depressor Action in Cats. Comparison of the depressor action of pure quinidine and dihydroquinidine was made in cats anesthetized with pentobarbital sodium. Blood pressure was recorded from the carotid artery. The drugs were administered intravenously. Owing to tachyphylaxis, only the first injection gave reliable results. Consequently, it was impossible to compare the depressor action of the 2 compounds in the same animal. These substances proved to have a marked depressor action only when given in comparatively large doses. In the case of dihydroquinidine, a dose of 10 mg. per kg. in 3 cats caused falls of blood pressure of 60, 110, and 75 mm. Hg, lasting 5, 23, and 20 minutes, respectively. An equal dose of pure quinidine to 2 cats resulted in blood pressure falls of 75 and 115 mm. Hg of 39+ and 44+ minutes' duration, respectively. A dose of 20 mg. per kg. produced a very rapid depressor effect and death in 3 to 4 minutes in 3 of 4 cats given pure quinidine, while 2 of 4 animals showed similar results with dihydroquinidine in the same dosage. Doses of 1 to 2 mg. per kg. of either substance produced only slight and very transient depressor effects. These results indicate little difference in the degree of depressor action of these 2 compounds in cats, except that the action of pure quinidine is more prolonged. Furthermore, doses necessary for marked depressor effect were considerably above those re-

TABLE 1

Comparative potency of pure quinidine, dihydroquinidine, and commercial quinidine sulfate in raising the threshold of permanent ventricular fibrillation induced by faradic stimulation

DRUG	CAT NUMBER	WEIGHT	DOSE	POSITION OF SECONDARY COIL IN RELATION TO PRIMARY	AVERAGE
None		kg.	mg. per kg.	cm.	
	1	3.487		8	9.25
	2	3.620		10	
	3	3.647		8	
	4	3.140		8	
	5	3.110		10	
	6	4.137		9	
	7	3.399		10	
	8	2.765		11	
Dihydroquinidine	9	3.665	0.50	7	10.33
	10	2.650		12	
	11	3.915		12	
	12	2.827	0.75	10	9.67
	13	4.189		12	
	14	3.277		7	
	15	3.300	1.00	6	5.67
	16	3.565		8	
	17	2.827		3	
	18	4.077	2.00	6	4.00
	19	3.270		3	
	20	2.815		3	
Pure quinidine	21	4.510	2.00	12	10.67
	22	3.350		9	
	23	4.548		11	
	24	4.347	3.50	7	4.40
	25	3.896		7	
	26	3.085		0	
	27	2.877		2	
	28	3.235		6	
	29	3.790	5.00	3	3.50
	30	3.500		4	
	31	4.164	10.00	0	0
Commercial quinidine sulfate	32	3.945	2.00	5	7.33
	33	3.312		9	
	34	3.412		8	
	35	3.120	5.00	3	2.50
	36	4.391		2	

quired to elevate the ventricular fibrillation threshold. In the report by Weisman (4), on the marked cardiovascular depressant action of pure quinidine in dogs, it is of interest to note that he used lethal doses (45 mg. per kg.) of quinidine and compared these effects with those resulting from doses of only 20 mg. per kg. of dihydroquinidine. Consequently, his conclusion that quinidine is a much stronger cardiovascular depressant than dihydroquinidine does not appear to be justified.

3. *Acute Toxicity in Mice.* The median lethal doses \pm standard errors were determined in albino mice by injection into the tail vein, for pure quinidine, dihydroquinidine, and commercial quinidine sulfate. Computations were made according to the method of Bliss (7). Results are given in table 2. Dihydroquinidine proved to be somewhat more toxic to mice than pure quinidine, the difference being statistically highly significant. Since commercial quinidine

TABLE 2

The acute toxicity of dihydroquinidine, pure quinidine, and commercial quinidine sulfate by intravenous injection into mice

DRUG	DOSE	NUMBER DIED NUMBER USED	LD ₅₀ \pm S. E.
	mg. per kg.		mg. per kg.
Dihydroquinidine	50	3/10	56.5 \pm 2.1
	56	3/10	
	62	8/10	
	80	5/5	
Pure quinidine	62	3/10	69.0 \pm 2.6
	70	4/10	
	80	9/10	
Commercial quinidine sulfate	50	0/5	73.5 \pm 2.8
	62	2/10	
	70	3/10	
	80	7/10	
	100	5/5	

sulfate is only 82.86% quinidine base, the figure for the base should be corrected to 60.9 \rightarrow 2.3 mg. per kg., a value between that of the other 2 substances.

After injection of pure quinidine, clonic convulsions appeared within a few seconds followed by death or recovery in 1 to 3 minutes. The onset of convulsions resulting from injection of dihydroquinidine occurred in 15 to 60 seconds, the convulsions being less intense but of somewhat longer duration than with pure quinidine.

Although the toxicity of dihydroquinidine in mice is about 18% greater than that of pure quinidine, the difference may not prove to be a disadvantage. In view of its stronger cardiac action, dihydroquinidine may be employed in smaller dosage, and the compound may actually prove safer for therapeutic purposes than quinidine.

These results confirm the earlier conclusions of Lewis and associates and Van

Dongen and Sanches that dihydroquinidine is more potent than pure quinidine or commercial quinidine sulfate against fibrillation of the heart. Further clinical trials of dihydroquinidine appear warranted.

SUMMARY

A comparison was made of some pharmacologic actions of pure quinidine, dihydroquinidine, and commercial quinidine sulfate, U.S.P. The following results were obtained:

1. The threshold doses of dihydroquinidine and quinidine required to raise the intensity of electrical stimulation needed to produce ventricular fibrillation in cats were approximately 1 and 3.5 mg. per kg., respectively.
2. In doses of 10 mg. per kg. intravenously into anesthetized cats, quinidine or dihydroquinidine caused a marked fall of blood pressure—almost of equal intensity. The duration of action of quinidine on blood pressure was more prolonged however. Doses of 1 to 2 mg. per kg. intravenously produced only slight and very transient fall of blood pressure.
3. From the median lethal doses obtained by intravenous injection into mice, dihydroquinidine was about 18% more toxic than quinidine.

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SOME EFFECTS OF TETRAETHYL AMMONIUM ON THE MAMMALIAN HEART

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The effect of the tetraethyl ammonium ion on the heart has been little studied. Trendelenburg (1) and others reported marked depression ("fast vollständiger Stillstand") of the excised frog heart with a 1 per cent concentration. This action of tetraethyl ammonium was not prevented by atropine, a fact which conforms with its generally accepted lack of muscarinic properties. Simon (2) reported that 7.8 millimols per liter of tri-tetraethyl ammonium phosphate "depresses" the isolated frog heart while the intravenous injection of 10 micromols per kgm. in the rabbit "excites" the heart.

Tetraethyl ammonium ion has some similarities to veratrum alkaloids in its action on nerve. Cowan and Walter (3) showed that tetraethyl ammonium causes isolated crab nerve to respond repetitively to stimulation with single, brief shocks, and that it decreases the threshold voltage for slowly rising currents. Both of these properties are shared by veratrine, as tested on circulated mammalian nerve (4, 5). Intra-arterial injections of 10 mgm. per kgm. of tetraethyl ammonium bromide into the leg circulation of the cat (6) produce the repetitive response to single, brief stimuli, the augmentation of negative afterpotential, and other related phenomena in the circulated peroneal nerve which veratrine also produces (5).

These similarities between tetraethyl ammonium ion and veratrum alkaloids with respect to nerve suggested the possibility of similarities in other systems. Veratrum alkaloids produce a recovery from failure in the heart-lung preparation of the dog, and in larger doses they cause cardiac irregularities. In the intact animal small doses lead to a marked bradycardia, a fall of arterial pressure and a complex respiratory response; and larger doses produce a discharge of epinephrine and a rise of arterial pressure (7, 8, 9).

The experiments with tetraethyl ammonium ion reported in this paper confirm the resemblance between this substance and veratrum alkaloids so far as a direct action on the mammalian heart is concerned. The bradycardia, the depressor response, the pressor response, and the effects on respiration and the discharge of epinephrine will be analyzed in a later paper.

METHODS Dog heart lung preparations were performed as described in previous communications (7). The animals weighed from 7 to 12 kgm and were anesthetized with sodium pentobarbital¹. Defibrinated blood was used. Tetraethyl ammonium bromide (Eastman Kodak Company) was kept in an aqueous stock solution of 10 per cent strength and diluted appropriately shortly before injection. Elementary analysis and negative tests for ash and amines showed that the sample of tetraethyl ammonium bromide used was practically

¹ Sodium pentobarbital (Nembutal) was kindly supplied by the Abbott Laboratories, North Chicago, Illinois

100 per cent pure.² The drug was usually added to the blood in the venous reservoir, but if a higher initial concentration in the heart was wanted, it was injected into the rubber tubing leading to the venous inflow cannula. The heart-lung system contained from 700 to 900 cc. of blood.

The observations on cardiac irregularities were made on the heart-lung preparations and on intact and vagotomized dogs and cats under anesthesia with sodium pentobarbital or 0.7 cc. per kgm. of Dial solution³ (each cc. containing diallyl barbituric acid [Dial], 0.1 gram, urethane, 0.4 gram, monoethyl urea, 0.4 gram, and distilled water q.s.) injected intraperitoneally. Electrocardiograms were taken with a 2-channel, inkwriting Grass oscillograph. Intravenous injections were made via a cannula in the jugular or femoral vein.

RESULTS: The action of tetraethyl ammonium bromide was tested in 20 heart-lung preparations. Three of these showed little evidence of failure at the time the substance was added to the inflowing blood. In 3 heart-lung preparations, "spontaneous" failure was allowed to occur before the drug was given. In the remaining preparations, the drug was tested after induction of failure by sodium pentobarbital (8 experiments), 2-naphthyl-1'-methylimidazoline hydrochloride (5 experiments)⁴ or quinacrine (1 experiment).

A. Effect on Work Capacity: Tetraethyl ammonium improves the work capacity of the heart-lung preparation. This effect is evident from a decrease of venous pressure, a decrease of heart volume, and an increase of systemic output. It is more specifically demonstrated by tests of the work capacity of the heart performed by increasing the blood supply (elevation of the level of blood in the venous reservoir by 50 or 100 mm.). Figure 1 illustrates these phenomena.

The improvement in work capacity is the more striking the more serious the heart failure. In the freshly prepared heart-lung preparations, a slight degree of failure may be assumed to have occurred, for tetraethyl ammonium, like the cardiac glycosides or the veratrum alkaloids, improves the heart's performance slightly. When the failure is severe, the improvement may be dramatic. No differences in the response of work capacity were noted between hearts failing as a result of the drugs listed above and the "spontaneously" failing heart.

The action is rapid in onset and reaches a maximum within a minute. With small doses (10 mgm.) it lasts for only 3 to 10 minutes, but with larger doses (100 mgm.) the effect may be apparent for 40 or more minutes. Ten milligrams is close to the minimal effective dose. In a total blood volume of 700 to 1000 cc., this represents a minimal effective concentration of 1:70,000 to 1:100,000, or 69 to 48 millimols per liter. Doses of 100 mgm. or more produce almost complete recovery from failure. Doses as high as 1 gram cause essentially the same effects unless the irregularities described below occur. Table 1 illustrates the effect of various doses upon the venous pressure and the systemic cardiac output of the failing heart.

² Tests done by Dr. Carl Tiedeke, New York.

³ Kindly supplied by Ciba Pharmaceutical Products, Summit, New Jersey.

⁴ Thanks are due to Miss Barbara Rennick and Ciba Pharmaceutical Products for the opportunity to test heart-lung preparations in which failure had been induced by 2-naphthyl-1'-methylimidazoline hydrochloride (Privine hydrochloride).

B. Effect on Heart Rate: In the three heart-lung preparations with little evidence of failure, the initial dose of 10 mgm. of tetraethyl ammonium bromide led to a temporary increase of 3 to 10 beats per minute from the basal rate of about 120 per minute. Subsequent larger doses caused longer lasting and greater tachycardia as a function of the dose. In one experiment, as a result of a series of doses of tetraethyl ammonium bromide totalling 1.34 grams given over a period of one hour, the heart rate rose stepwise from 112 to 202 per minute. After each dose, the heart rate rose to a peak in the first minute after the injection

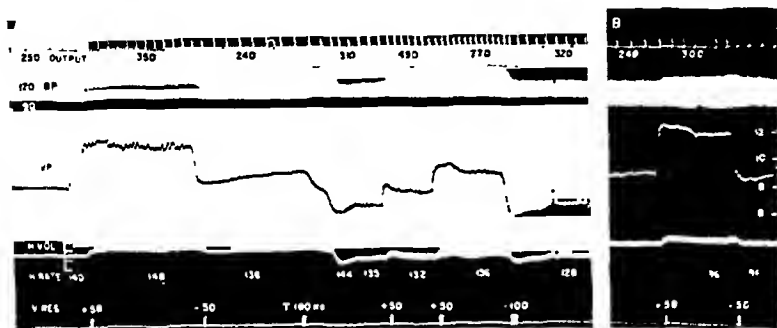


FIG. 1. THE EFFECT OF TETRAETHYL AMMONIUM BROMIDE ON THE FAILING HEART. Heart-lung preparation, dog, 7 kgm. Tracings, from top to bottom: systemic output of the heart signaled in 100 cc. volumes (Weese stromuhr); time in 10-second intervals; arterial pressure (mercury manometer), scale at left in mm. Hg; venous pressure from the right auricle (water manometer), scale at right in cm. of water; volume of the heart from cardiometer, each division of the scale representing a change of 10 cc.; signal.

Failure, induced by sodium pentobarbital, is evident from the low output and high venous pressure at the beginning of the record. The figures over the signals indicate the mm. through which the venous reservoir were elevated and lowered in tests of the work-capacity of the heart by changing the blood supply to the right heart. In the early part of the record, raising the reservoir 50 mm. led to only a moderate increase of cardiac output and a sizable increase in venous pressure.

At T, 100 mgm of tetraethyl ammonium bromide was introduced into the venous reservoir. The blood volume of the entire system was about 700 cc. The heart responded by an increase of output and work capacity, and a decrease of venous pressure and heart volume. The heart rate changed insignificantly.

Between A and B, there was a pause of 22 min. In B, it is evident that the failure has returned

into the venous blood and then declined slowly during the 5 to 15 minutes before the next dose.

In the failing hearts, the heart rate was affected much as in those which were tested before serious failure had set in. When a pause of 20 to 40 minutes elapsed after a dose of tetraethyl ammonium in hearts failing from treatment with sodium pentobarbital, the initial cardiac acceleration gave place to a gradual deceleration below the basal rate (fig. 1). In at least some of these experiments, the bradycardia was of ventricular origin, with auriculo-ventricular dissociation of varying degree; the auricles were beating faster than normal. Similarly, in three experiments in anesthetized intact cats, slowly progressive poisoning by successive intramuscular or intraperitoneal injections totalling about 30 mgm.

per kgm. of tetraethyl ammonium bromide produced progressive bradycardia unaffected by vagotomy.

C. *Cardiac Irregularities:* In the denervated heart-lung preparation of the dog and in the intact anesthetized dog or cat, large doses of tetraethyl ammonium bromide produce a number of alterations of the production and transmission of cardiac impulses. An intravenous injection of 25 or more mgm. per kgm. in the intact dog or cat, or a dose of 100 or more mgm. in the heart-lung preparation is regularly followed by a characteristic change in the electrocardiogram, as illustrated in figure 2. This consists of a change in the amplitude of the T-wave, usually in such a way as to lead to a large T-wave in the same direction as the P-wave when the leads are in a position comparable to lead I of the human electro-

TABLE 1

The effect of the concentration of tetraethyl ammonium ion on the failing heart-lung preparation of the dog, as measured by the response of venous pressure and systemic cardiac output

Venous pressure response is calculated as:

$$100 \times \frac{\text{Decrease in venous pressure due to tetraethyl ammonium}}{(\text{Failure level of venous pressure}) \text{ minus } (\text{Initial level})}$$

Systemic output response is calculated as:

$$100 \times \frac{\text{Increase in systemic output due to tetraethyl ammonium}}{(\text{Initial level of systemic output}) \text{ minus } (\text{Failure level})}$$

DOSE MGm. OF TEA Br.	CONCENTRATION OF TEA Br.	MICROMOLS PER LITER	PER CENT RECOVERY FROM FAILURE	
			Venous pressure response	Systemic Output response
10	1:90,000	53	9	22
10	1:87,000	55	13	8
30	1:29,000	160	42	48
50	1:18,400	260	27	39
50	1:17,400	270	57	66
100	1:8,700	550	94	87
100	1:8,500	560	70	76
100	1:8,500	560	57	77
150	1:6,500	740	110	121

cardiogram. Associated with this is usually a change in the S-T segment, which is no longer isoelectric, but slopes from a high or low position at the end of the S-wave to the beginning of the augmented T-wave. The electrocardiogram returns to normal in 2 to 6 minutes. In no instance was there a prolongation of the P-R interval.

Larger doses of tetraethyl ammonium bromide lead to ventricular extrasystoles, singly, in groups alternating with groups of normal beats, or in the form of continuous ventricular tachycardia. With sufficiently large doses, ventricular fibrillation results. Occasionally, the A-V node became the pacemaker. In heart-lung preparations failing as a result of sodium pentobarbital, temporary auricular asystole or A-V dissociation follow doses of 200 or more mgm. of

tetraethyl ammonium. In one of the experiments in cats mentioned in section B, in which successive intramuscular or intraperitoneal injections were given, the bradycardia suddenly changed to pulsus bigeminus.

In the hearts with little evidence of failure, in the experiments in which "spontaneous" failure was allowed to occur, and in the hearts in which failure was induced by 2-naphthyl-1'-methylimidazoline hydrochloride, 200 mgm. of tetraethyl ammonium bromide caused no irregularities and doses as high as 500



FIG 2 THE EFFECT OF A LARGE INTRAVENOUS DOSE OF TETRAETHYL AMMONIUM BROMIDE ON THE ELECTROCARDIOGRAM

Dog, 16 kgm, anesthetized with sodium pentobarbital. Vagi cut. At the left, the interval, in sec, after the injection of 31 mgm per kgm. into the jugular vein; at the right, the corresponding strips of ECG, recorded with an inkwriting oscillograph.

or 1000 mgm. did not uniformly cause irregularities. Sodium pentobarbital, however, favors the appearance of cardiac irregularities in the heart-lung preparation when tetraethyl ammonium bromide is given. In the hearts failing as a result of treatment with sodium pentobarbital, 200 mgm. uniformly caused extrasystoles, tachycardia, or fibrillation of ventricular origin. If a given dose was injected into the venous inflow cannula, so that the concentration reaching the heart was initially high, the irregularities occurred rapidly and for a short

time. If, on the other hand, the drug was admitted to the heart slowly, greater doses could be administered without the occurrence of irregularities.

DISCUSSION: Tetraethyl ammonium ion has a positive inotropic effect on the failing heart-lung preparation of the dog. In larger doses it causes a variety of irregularities of the heart. This combination of properties places it, pharmacologically speaking, in the class of substances of which the cardiac glycosides form an important group. In the same respects, it resembles those veratrum alkaloids which have been studied in the manner described in this paper (7, 8, 9). Yet its chemical structure is radically different from that of either of these groups of compounds.

Previous pharmacological data on tetraethyl ammonium ion consist largely of negative findings (1). Other members of the large group of quaternary ammonium compounds exhibit curariform, nicotinic, muscarinic and anti-muscarinic properties. Tetraethyl ammonium has been found to lack the muscarinic property, its possession of curariform activity has been denied (10), and the evidence for anti-muscarinic properties is flimsy. Data which are to be published in a subsequent paper show that tetraethyl ammonium bromide injected intravenously in anesthetized cats and dogs causes a marked depressor response and a moderate bradycardia, both of which may be explained by a rather specific blockage of autonomic ganglia while much larger doses cause a pressor response accompanied by the discharge of epinephrine.

No cardiac effects other than the muscarinic one have previously been attributed to any of the quaternary ammonium compounds except tetraethyl ammonium; and the reports concerning the cardiac effects of the latter have hardly been taken seriously. The properties of tetraethyl ammonium (positive inotropic effect and cardiac irregularities) described in this paper are, therefore, new and unique for compounds of this group.

The members of the lanata group of cardiac glycosides were analyzed by Moe and Visscher (11) for two important variables: namely, the concentration producing the positive inotropic effect ("minimal efficiency dose"), and the concentration producing cardiac irregularities ("minimal irregularity dose"). The same analysis has been carried out for veratrum alkaloids in this laboratory (8, 9). These studies reveal that these properties, which appear to be linked together in these and other drugs having cardiac action, are independently variable so far as effective concentration is concerned. Thus, the ratio of minimal positive inotropic concentration to minimal toxic concentration is 1 to 12 for veratridine, 1 to 5 for veratrine, and 1 to 3 for protoveratrine.

For tetraethyl ammonium bromide this ratio is difficult to determine accurately because of the relative brevity of the effects of the drug. Thus, unlike the cardiac glycosides, tetraethyl ammonium ion has only a transient effect on the failing heart, lasting 10 to 40 minutes. Moreover, quick injection of a large dose is more likely to lead to cardiac irregularities than slow or divided injection of the same dose. From the data given above, a rough approximation of the ratio, minimal positive inotropic concentration to minimal toxic concentration, is 1 to 20 in hearts failing as a result of treatment with sodium pentobarbital.

If, however, the data on hearts failing from other causes are considered, this ratio becomes 1 to 100.

SUMMARY: 1. Tetraethyl ammonium bromide, in concentrations from 1:100,000 (48 micromols per liter) upward, improves the work capacity of the heart in the heart-lung preparation of the dog. This improvement is slight in hearts with little evidence of failure, but is the more striking the more severe the heart failure. It occurs whether the failure is "spontaneous," or whether it is induced by sodium pentobarbital, 2-naphthyl-1'-methylimidazoline hydrochloride (Privine), or quinacrine.

2. Concentrations from 1:100,000 upward lead to slight to moderate acceleration of the rate of the denervated heart, followed by a slow progressive deceleration below the basal rate.

3. Concentrations of 1:10,000 upward produce changes in the T-wave and the S-T segment of the electrocardiogram, and larger doses produce ventricular extrasystoles or fibrillation.

4. The dose which produces cardiac irregularities is 200 mgm. when failure has been induced by sodium pentobarbital, but 1 gram in the other conditions studied.

5. In intact anesthetized dogs and cats, intravenous doses of 25 or more mgm. per kgm. produce the same changes in the T-wave and the S-T segment of the electrocardiogram, extrasystoles of the same nature, and the same slow deceleration of the heart as those observed in the heart-lung preparation.

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ERRATUM

The title of the article by Heppel et al. appearing on page 53 of the May issue should read "The Toxicology of 1,2-Dichloroethane (Ethylene Dichloride)."

In the article by Chance and Lobstein on "The Value of the Guinea-Pig Corneal Reflex for Tests of Surface Anaesthesia" published in this journal, 82, 203, 1944, on page 206 under Volume of Doses, in Dr. Lesser's experiments the anesthetic was injected beneath the conjunctiva. The results then are not comparable to those in which it was applied to the surface and involve passage through an epithelial membrane.

PHARMACOLOGIC STUDIES OF A NEW VASOCONSTRICTOR: 2-NAPHTHYL-(1')-METHYL-IMIDAZOLINE HYDROCHLORIDE (PRIVINE OR NAPHAZOLINE)¹

III. AN ATTEMPT TO LOCALIZE THE SEAT OF ACTION IN TERMS OF ADRENERGIC AND CHOLINERGIC FOCI

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In a previous communication (1) evidence was presented which indicated that 2-naphthyl-(1')-methyl-imidazoline HCl (Privine) acts more like ephedrine than epinephrine. Although privine somewhat resembles the latter by being mildly potentiated by cocaine, it differs from epinephrine in not having its action reversed by the adrenolytic and sympatholytic agents ethyl yohimbine (2, 3) or piperidinomethyl benzodioxane, 933F (4). In this respect it resembles vasoconstrictors related to ephedrine.

It seemed desirable to use other test objects or functions in an effort to further elucidate the action of this synthetic agent (5), and the procedure to be described was adopted.

Method. Twenty-four cats of both sexes were anesthetized with either urethane, ether or dial-urethane and were surgically prepared for insertion of salivary, carotid and venous cannulae. Wharton's duct of the submaxillary gland was selected for cannulation and the ipsilateral chorda tympani and cervical sympathetic nerves were arranged for faradization after the vagi were sectioned. Salivary flow was recorded on a horizontal volumometer calibrated to register one centimeter as representing 0.0345 cc. The ipsilateral nictitating membrane was needled and arranged for kymographic registration of its excursions simultaneously with carotid arterial pressure which was recorded through a mercury manometer. Pupillary changes were measured with a millimeter rule. All faradization was effected with a Harvard inductorium. Several drugs of varying concentrations as described below were injected intravenously and the sequence of administration was altered to satisfy the intent of the investigation, namely, to localize the seat of action of privine. These drugs included pituitrin, the hydrochlorides of privine, epinephrine, ephedrine, ethyl yohimbine³ and pilocarpine, and the sulphate of atropine. Privine was occasionally administered intraperitoneally, orally, rectally or directly into the stomach or small intestine. All doses cited are in terms of milligrams per kilogram.

RESULTS. Privine, 0.1 mgm., was effective as a vasoconstrictor in cats as well as in dogs (1). However, it seemed to be less effective than epinephrine, which

¹ This contribution was initiated at Wayne University College of Medicine, Detroit, Michigan. It represents part of the project supported at that institution by Ciba Pharmaceutical Products, Inc., in the form of a research grant established for the investigation of new synthetic vasoconstrictors, including Privine.

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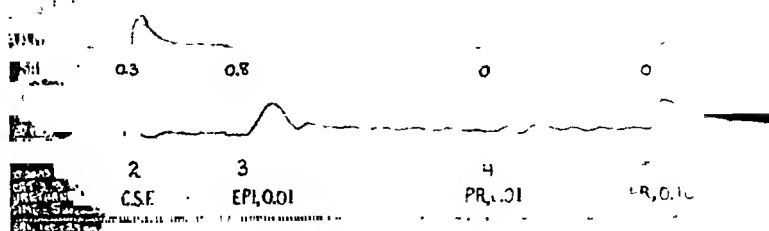


FIG. 1. CAT. 2.3 KG., 10/20/43. URETHANE ANESTHESIA

In descending order, right nictitating membrane function, right submaxillary salivation expressed in centimeters, left carotid blood pressure, time in five seconds.

1. Vagi sectioned; 2. Cervical sympathetic nerve faradized for 10 seconds (C.S.F.).
3. Epinephrine, 10 gamma; 4. Privine, 10 gamma; 5. Privine, 100 gamma.

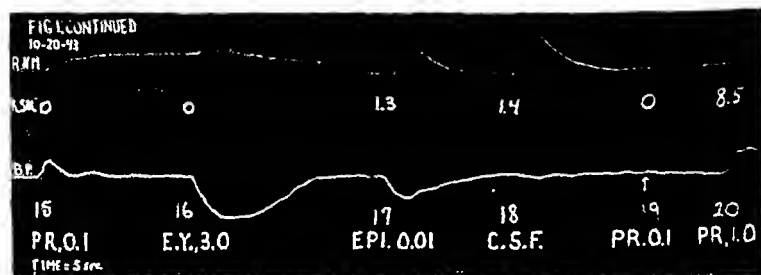


FIG. 2.

Continuation of figure 1. Time lapse of 70 minutes between 5 and 15, during which C.S.F., epinephrine and ephedrine were exercised.

15. Privine, 100 gamma; 16. Ethyl yohimbine, 3 milligrams; 17. Epinephrine, 10 gamma; 18. Cervical sympathetic nerve faradized for 10 seconds; 19. Privine, 100 gamma; 20. Privine, 1 milligram.

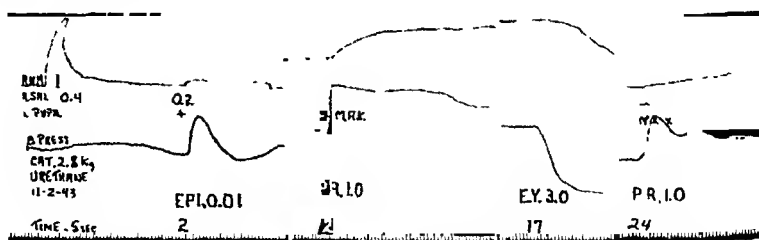


FIG. 3. CAT. 2.8 KG., 11/2/43. URETHANE ANESTHESIA

In descending order, right nictitating membrane function, right submaxillary salivation, reaction of left pupil with + denoting dilatation, - no change, and MAX representing maximal dilatation, left carotid blood pressure, time in five seconds.

1. Cervical sympathetic nerve faradized for 10 seconds.
2. Epinephrine, 10 gamma. Time lapse of approximately 50 minutes between 2 and 12, during which privine, ephedrine, pilocarpine, atropine and chorda tympani faradization (C.T.F.) were exercised.
12. Privine, 1 milligram. Time lapse of approximately 20 minutes between 12 and 17, during which C.T.F., C.S.F., pilocarpine and epinephrine were exercised.
17. Ethyl yohimbine, 3 milligrams. Time lapse of approximately 15 minutes, during which pituitrin, epinephrine and C.S.F. were exercised.
24. Privine, 1 milligram

produced a comparable rise in blood pressure with a dose of only 0.01 mgm. (fig. 1).

Privine was effective (fig. 2 and 3), although less than normally, in elevating and sustaining tension after ethyl yohimbine, 3.0 mgm. The effect of privine was not reversed as was characteristic of epinephrine following the yohimbine radical.

Privine produced no salivary secretion until a concentration of 1.0 mgm. was used (fig. 2) and this secretion was arrested by atropine, 0.2 mgm. but not by ethyl yohimbine, 3.0 mgm. (fig. 4).

Privine invariably caused a retraction of the nictitating membrane, even after rectal insertion of such a small dosage as 0.2 mgm. This effect was partially nullified by atropine and likewise by ethyl yohimbine (figs. 4, 5, and 6). Some contraction of the nictitating membrane frequently resulted after either atropine or ethyl yohimbine but seemed not to be maximal (fig. 3). The nictitating mem-

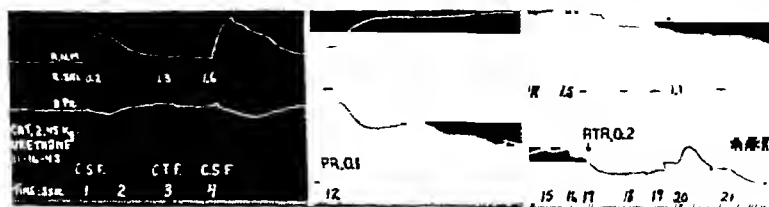


FIG. 4. CAT. 2.5 Kg. 11/16/43 URETHANE ANESTHESIA

In descending order, right nictitating membrane function, right submaxillary salivation, left earotid blood pressure, time in five seconds

1. Cervical sympathetic nerve faradized for 10 seconds 2 Vagi sectioned 3. Chorda tympani nerve faradized for 10 seconds 4 Same as 1 Time lapse of approximately 25 minutes between 4 and 12, during which privine in varying doses was administered enterally 12. Privine, 100 gamma. Time lapse of 18 minutes between 12 and 15 during which certain unknowns not germane to this presentation were exercised 15. & 18 Chorda tympani nerve faradized for 10 seconds 16 & 19 Cervical sympathetic nerve faradized for 10 seconds. 17. Atropine, 0.2 milligrams 20 Epinephrine, 50 gamma. 21. Ethyl yohimbine, 3 milligrams

brane was the most sensitive indicator of privine's action since intravenous dosages as low as from 0.001 to 0.01 mgm. could retract it (fig. 6).

Privine produced no change in any function studied after administration of 1 mgm. into the stomach or of 0.5 mgm. into the ileum, but it did elevate blood pressure and retract the nictitating membrane when given rectally, 0.6 mgm. or less, or intraperitoneally, 0.5 mgm.

Pupillary reactions were sufficiently variable to preclude any unequivocal conclusion to be drawn therefrom; predominantly, however, the pupil responded by dilating rather than by constricting.

DISCUSSION. These experiments indicate that the hypertensive action of privine in the cat is, as shown previously in the dog (1), similar to ephedrine's pressor action rather than to that of epinephrine, since its effect is not reversed by ethyl yohimbine. This fact, however, does not preclude the possibility of privine's possessing other sympathomimetic capacities similar to those of epinephrine. The method described above was chosen so that dually innervated

organs could be studied simultaneously. Unfortunately, in a sense, for our purposes, the sphincter pupillae, nictitating membrane and submaxillary gland are controlled by three different cranial nerves, namely, the third, sixth and seventh respectively, but on the other hand, a balancing innervation for the functions involved is singularly afforded by the cervical sympathetic nerve. If prinine possessed any sympathomimetic properties they should have been manifested in either mydriasis, salivation or retraction of the nictitating membrane. Likewise,

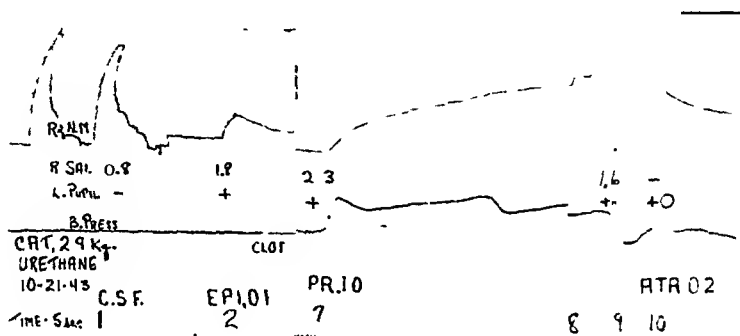


FIG. 5. CAT. 2.3 Kg., 10/21/43. URETHANE ANESTHESIA

In descending order, right nictitating membrane function, right submaxillary salivation reaction of left pupil, + or -, left carotid blood pressure, time in five seconds.

1. Cervical sympathetic nerve faradized for 20 seconds. 2. Epinephrine, 100 gamma. Time lapse of approximately 15 minutes, between 2-7. 7. Prinine, 1 milligram. 8. Time lapse of 7 minutes. 9. Pilocarpine, 1 milligram. 10. Atropine, 0.2 milligram.

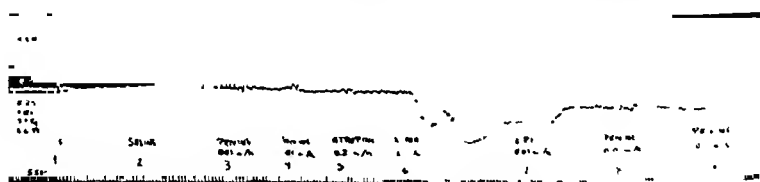


FIG. 6. CAT 3.3 Kg., 6/6/44 DIAL-URETHANE ANESTHESIA

In descending order, right nictitating membrane function, left carotid blood pressure, time in five seconds.

1. Cervical sympathetic nerve faradized for 10 seconds; 2. Physiologic sodium chloride solution, 2 cc.; 3. Prinine, 1 gamma; 4. Prinine, 10 gamma; 5. Atropine, 200 gamma; 6. Ethyl yohimbine, 3 milligrams; 7. Epinephrine, 10 gamma; 8. Prinine, 10 gamma; 9. Prinine, 100 gamma.

cholinergic or parasympathomimetic potentialities could have become evident by miosis, salivation or lateral protrusion of the nictitating membrane, which latter function, according to the excellent work of Rosenblueth & Bard (6) is controlled by the sixth cranial nerve.

The important effects of prinine are illustrated in figure 1. In this experiment no salivary secretion is induced by 0.1 mgm. but the nictitating membrane retracts and some increase in carotid tension occurs,—a result which could be caused by adrenergic stimulation. However, 1.0 mgm., usually initiates a

slightly delayed but marked salivary flow which is arrested only by atropine (fig. 2). This response demonstrates the cholinergic property of the *larger* doses of privine and is a property which was not anticipated. The increased secretion was not prevented by adrenolytic amounts of ethyl yohimbine, which fact supports the conclusion that as a sialogogue privine in this dosage is cholinergic rather than adrenergic. Further evidence validating this conclusion is afforded by the fact that faradization of the cervical sympathetic nerve results in renewed salivary flow, after salivation promoted by privine has been inhibited by atropine. In such a condition, with the animal under the influence of atropine but not pretreated with yohimbine, epinephrine acts in the same manner as faradization of the cervical sympathetic nerve, but pilocarpine, acetylcholine and faradization of the chorda tympani do not. Thus, in reference to salivation, a large dose of privine apparently acts cholinergically like pilocarpine and acetylcholine but not adrenergically like epinephrine or sympathin.

It seems that the nictitating membrane is one of the most consistently sensitive indicators of privine's actions that have been studied to date. Amounts of the drug as small as 0.01 mgm. or less produce a slow, gradual retraction which, if given time, may become almost maximal. Retraction by privine may occur after atropine administration although here it is usually not maximal; more significantly, atropine relaxes in part a privine-induced contraction of muscle in the nictitating membrane. This effect indicates perhaps some cholinergic activity of privine in this muscle, an effect which was not anticipated since the only known neural components of cranial origin in the nictitating membrane arise from the sixth cranial nerve and, according to Rosenblueth and Bard (6), faradization of this nerve results not in retraction but rather in extrusion of the membrane laterally across the cornea and this result is registered by a fall rather than a rise in the recording lever. The same result would be anticipated if the end organs of the sixth cranial nerve were cholinergically reactive. However, retraction rather than extrusion of the nictitating membrane, followed the administration of privine. The same result has been observed with pilocarpine by others and may indicate merely, as Rosenblueth (7) suggests, that the adrenergically reactive components associated with the cervical sympathetic nerve in this muscle are also reactive to certain cholinergic effectors and apparently more so than those associated with a true cranial efferent nerve such as, in this instance, the sixth.

There seems to be less question regarding the explanation of ethyl yohimbine's capacity to annul that component of retraction of the nictitating membrane that is induced by privine. Ethyl yohimbine augments or completes, as a rule, atropine's nullification of privine retraction (figs. 5 and 6). Extremely large doses of ethyl yohimbine are required for sympatholysis but less for lysis of privine's effect. This was also true of epinephrine or adrenaline (2), and it would seem that privine is perhaps somewhat cholinergic and also partially, but definitely adrenergic in eliciting a retraction of the nictitating membrane, since this retraction is in part lysed by atropine and more completely so by ethyl yohimbine.

The available evidence indicates that pupillary dilatation usually follows administration of privine and one would believe this reaction to be adrenergic rather than anticholinergic in nature. This seems reasonable since it is difficult to com-

prehend a simultaneous cholinergic action of privine as a sialogogue and an anticholinergic action as a mydriatic.

On the basis of results presented it can be concluded that privine, although cholinergic in some of its actions, is only weakly so in relation to salivation since large doses of the drug are required to initiate salivary flow. It seems to be also somewhat cholinergic in retracting the nictitating membrane since small amounts, regardless of how administered, are effective in this respect. In this situation, it must not be forgotten, however, that retraction is powerfully augmented or facilitated by the concomitant adrenergic powers of privine.

The import of privine's action following administration by routes other than the intravenous is discussed elsewhere (1). The results reported here indicate that there is no species difference between the cat and the dog in this respect.

The mixture of cholinergic and adrenergic actions demonstrated by privine indicates the undesirability of classifying an agent such as privine solely as a vasoconstrictor drug, even though such a capacity seems at present to be its chief characteristic. Its other pharmacologic properties, such as its cholinergic potentialities, must not be ignored, not only for purposes of pharmacologic classification but more particularly so when its possible substitution for ephedrine is considered in certain clinical situations such as hypotension, bronchial asthma, or associated conditions requiring prolonged ephedrine-like medication.

CONCLUSIONS

1. Privine, in adequate dosage in anesthetized cats can simultaneously elevate arterial tension, retract the nictitating membrane, induce salivation and dilate the pupil. As a hypertensive agent privine, however, is less potent than epinephrine.

2. Retraction of the nictitating membrane seems to be induced both cholinergically and adrenergically since it is lysed by both atropine and ethyl yohimbine. Privine, regardless of its route of administration, is perhaps most consistently detected physiologically in very small amounts by this sensitive indicator, the nictitating membrane.

3. Salivary secretion is induced only by large doses of privine, a reaction that is cholinergic in nature since it is completely nullified by atropine.

4. Dilatation of the pupil by privine is probably induced adrenergically.

5. The varied pharmacologic effects of privine observed experimentally suggest that under certain conditions and in excessive amounts this drug may invoke cholinergic as well as adrenergic reactions.

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THERAPEUTIC ACTIVITY AND TOXICITY OF SOLUTHIAZOLE AND SOLUPYRIDINE

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The therapeutic activity of the sulphonamides depends largely on obtaining adequate blood concentrations, and since there are irregularities in absorption from the gut, supplementary parenteral administration is often indicated, particularly at the beginning of treatment.

Except for neutral soluble derivatives of sulphanilamide which are in use, the only injectable sulphonamides at present available are the sodium salts, which are, apart from sodium sulphacetamide, highly alkaline and cause local tissue damage. The addition of a group to increase solubility tends to reduce therapeutic activity, but in view of the clinical use of "Soluseptasine", a neutral soluble derivative of sulphanilamide, the analogous derivatives of sulphapyridine and sulphathiazole were prepared. These we have called solupyridine and soluthiazole respectively, and they are the disodium cinnamylidene dibisulphite derivatives of the parent compounds. Their molecular weights are 571 and 577, and 1 g. contains respectively 0.434 g. and 0.442 g. of sulphapyridine and sulphathiazole. Both substances are readily soluble in water, and we have used solutions containing 20 per cent of the parent sulphonamide. These solutions have a pH value of about 8, and can be sterilised by autoclaving in sealed containers.

On the assumption that these compounds owe their activity to the liberation in the body of their parent sulphonamides, all dosage and concentration figures are given in terms of the parent compounds.

THERAPEUTIC ACTIVITY. The compounds were tested against *Streptococcus pyogenes*, *Staphylococcus aureus* and *Pneumococcus* (Type I).

In vitro tests. When tested *in vitro* against *Staphylococcus aureus* (two strains) by Fleming's slide-cell technique, using human defibrinated blood, the average minimal effective concentration for a number of estimations was 1:4,000 for soluthiazole as compared with 1:32,000 for sulphathiazole. Solupyridine was similarly less active than the parent compound. Although, however, these results did not appear promising, it was felt that *in vivo* experiments would provide a more useful evaluation.

In vivo experiments. In designing these experiments, we took into account the absorption and excretion of the compounds. Schmidt, Sesler and Hughes (1) and Marshall, Litchfield and White (2) have shown the importance of evaluating activity on blood concentrations, instead of on the doses employed. Since we found, however, that the absorption and excretion of solupyridine and soluthiazole were similar, a reliable comparison of the potencies could be based on the effective doses.

Albino mice of a laboratory-bred strain were used throughout the experiments.

Streptococcus pyogenes. Infection was by intraperitoneal injection of a broth dilution of an 18-hour culture. Administration of the compounds by the subcutaneous route was begun one hour after infection, and repeated once daily for seven days. The sodium salts of the parent compounds were used as controls.

Two experiments were carried out. The infective dose in the first experiment was 0.25 cc. of a 10^{-4} dilution, and in the second experiment, after the virulence had been increased by passage, 0.5 cc. of a 10^{-6} dilution. Groups of 10 mice were used on each dose. The results of the second experiment are given in table 1.

TABLE 1

The effect of soluthiazole and solupyrindine against streptococcal infections in mice
Injections once daily

COMPOUND	DOSE mg./g.	NO. OF MICE	NO. OF SURVIVORS EACH DAY							EXPECTA- TION OF LIFE days
			1	2	3	4	5	6	7	
Control		10	1	0	0	0	0	0	0	0.6
Sodium sulpha- pyridine	0.5	10	10	10	10	7	7	7	7	5.95
	0.25	10	10	10	9	8	6	3	1	5.15
	0.125	10	10	6	4	2	2	0	0	2.9
	0.0625	10	10	9	2	1	0	0	0	2.7
Solupyrindine	0.5	10	10	10	10	10	10	10	9	6.95
	0.25	10	10	10	8	8	8	8	5	5.95
	0.125	10	10	7	5	3	2	1	1	3.35
	0.0625	10	10	6	3	3	1	1	1	2.95
Sodium sulpha- thiazole	0.5	10	10	9	7	7	6	5	4	5.1
	0.125	10	10	2	2	1	1	0	0	2.1
	0.0625	10	9	1	0	0	0	0	0	1.5
Soluthiazole	0.25	10	10	6	6	6	5	5	5	4.55
	0.125	10	10	5	3	3	1	1	1	2.85
	0.0625	10	10	1	0	0	0	0	0	1.6

These results are also shown graphically in fig. 1. The expectation of life is based on a formula used by Goodwin (3). This was then plotted against the logarithm of the dose and a line fitted graphically to the steepest part of the curve. The positions of maximum response (7 days) and zero response (expectation of life of the control group) were marked on the graph, and the dose corresponding to an expectation of life midway between these two extremes was recorded as the standard effective dose (S.E.D.).

In the second experiment shown in fig. 1, the standard effective doses for sodium sulphapyridine, solupyrindine, sodium sulphathiazole and soluthiazole were respectively 0.17, 0.15, 0.25 and 0.19 mg./g. The results obtained in the first experiment were plotted graphically in the same manner, and the figures

were respectively 0.12, 0.16, 0.23 and 0.25 mg./g. Both soluthiazole and solupyrindine were as active as the corresponding sodium salts, and solupyrindine appeared slightly more effective than soluthiazole.

Staphylococcus aureus. We obtained a strain of staphylococcus which gave a lethal infection in mice without using mucin. An 18-hour blood broth culture was diluted to give 400,000 organisms per cc., and 0.5 cc. was given intraperitoneally to each mouse. Groups of 10 mice were used on each dose. The injections began subcutaneously one hour after infection, and were repeated for ten days. The results are given in table 2.

TABLE 2

The effect of soluthiazole and solupyrindine against staphylococcal infections in mice
Injections once daily

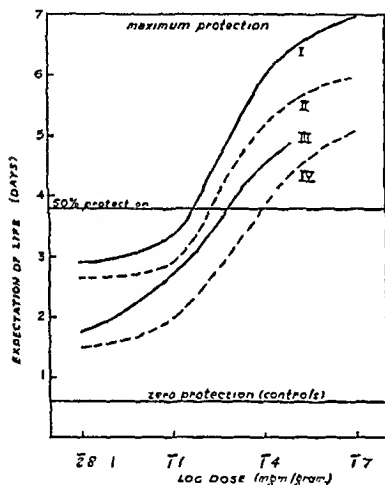
COMPOUND	DOSE mg./g.	NO. OF MICE	NO. OF SURVIVORS EACH DAY										EXPEC- TATION OF LIFE days
			1	2	3	4	5	6	7	8	9	10	
Control		10	0	0	0	0	0	0	0	0	0	0	0.5
Sodium sulpha- pyridine	0.5	10	9	9	9	9	9	9	9	9	8	6	8.8
	0.25	10	6	6	6	6	2	1	1	0	0	0	3.3
	0.125	10	3	3	3	3	1	0	0	0	0	0	1.8
Solupyrindine	1.0	10	10	10	10	10	10	9	9	9	8	6	9.3
	0.5	10	9	8	4	4	3	2	0	0	0	0	3.5
	0.25	10	4	4	4	3	1	0	0	0	0	0	2.1
	0.125	10	5	2	2	1	1	0	0	0	0	0	1.6
Sodium sulpha- thiazole	0.5	10	10	10	9	9	9	8	6	6	5	5	8.0
	0.25	10	6	3	3	3	0	0	0	0	0	0	2.0
	0.125	10	3	2	2	1	0	0	0	0	0	0	1.3
Soluthiazole	1.0	10	10	10	10	9	9	8	4	4	4	4	7.5
	0.5	10	7	5	1	1	1	0	0	0	0	0	2.0
	0.25	10	5	2	2	1	1	1	1	0	0	0	1.8

These results, plotted graphically, are shown in fig. 2. The standard effective doses for sodium sulphapyridine, solupyrindine, sodium sulphathiazole and soluthiazole were respectively 0.32, 0.58, 0.37 and 0.74 mg./g. Since the curves are approximately parallel, the potencies can be compared from a consideration of the standard effective doses. On this basis, both the sodium salts were about twice as active as the new compounds, thus confirming the *in vitro* experiments with *Staphylococcus aureus*. The analogues were therefore less effective against staphylococcal than against streptococcal infections. These results also showed solupyrindine to be slightly more effective than soluthiazole, although we do not attach much significance to this difference.

Pneumococcus (Type I). The inoculum was 0.5 cc. of an 18-hour blood broth culture diluted 10^{-4} or 10^{-7} according to its virulence. Because the compounds

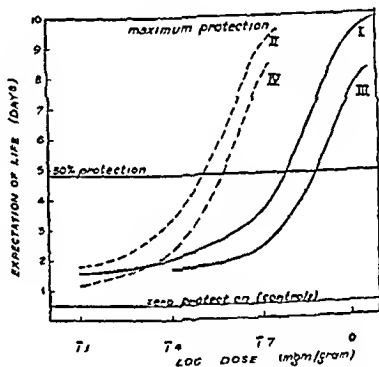
were excreted very rapidly we found it necessary to give subcutaneous injections every three hours during the day and night for four days, and as the injections had to be given continuously during the 24 hours, we did not attempt to obtain a graded dose-response curve, although the results showed that on equal doses similar effects were observed with the two compounds. Mice surviving after the fourth day were observed for a further six days for relapse. The results are given in table 3.

Since a possible use of these derivatives would be the production by injection of an initial high concentration in the blood, followed later by oral administration of the parent compounds, a series of experiments was designed on the following



- I. Solupyrindine
- II. Sodium sulphapyridine
- III. Soluthiazole
- IV. Sodium sulphathiazole

FIG. 1. STREPTOCOCCAL INFECTIONS IN MICE



S.E.D. (Mg/g.)	
Fig 1	Fig 2
0.15	0.55
0.17	0.32
0.19	0.74
0.25	0.37

FIG. 2. STAPHYLOCOCCAL INFECTIONS IN MICE

lines. The concentration in the blood was maintained for the first seven hours after infection by repeated subcutaneous injections of 0.6 mg./g. at two-hourly intervals of the soluble compound, followed by sub-maximal doses (0.5 mg./g.) by mouth of the parent substances every 24 hours for four days. This treatment (a) was compared with (b) a series of mice in which the oral administration of the parent compound was delayed up to seven hours, and no injections of the soluble compound were given. It was found that for sulphapyridine the expectation of life for treatments (a) and (b) was 4.7 and 3.7 days respectively, and for sulphathiazole 4.4 and 3.1 days. Initial treatment with the soluble derivatives, therefore, was advantageous

TOXICITY. Systemic toxicity. The toxicities of the disodium cinnamylidene dibisulphite derivatives were accurately determined in comparison with the sodium salts of the parent compounds. The compounds were given by intravenous, subcutaneous and oral administration, and mortalities were observed for a period of seven days. The results were calculated statistically by Gad-

TABLE 3

The effect of solupyrindine and soluthiazole against pneumococcal (Type I) infections in mice

Injectons every 3 hours for 4 days

COMPOUND	DOSE	NO. OF MICE	NO. OF SURVIVORS EACH DAY										EXPECTATION OF LIFE	
			1	2	3	4	5	6	7	8	9	10	During treatment	After treatment
	mg./g.												days	days
Control.....		10	9	1	0	0	0	0	0	0	0	0	1.5	
Solupyrindine...	0.6	10	9	9	9	9	9	8	8	7	7	6	3.65	3.3
Soluthiazole.	0.6	10	10	10	10	10	9	6	5	5	4	4	4.0	2.75

TABLE 4

The acute toxicities in mice of solupyrindine and soluthiazole in comparison with the sodium salts of the parent compounds

COMPOUND	METHOD OF ADMINISTRATION	NO. OF MICE	L.D. 50	$b \pm \sigma b$
Solupyrindine	Intravenous	80	mg./g. 1.28	14.9 ± 3.7
	Subcutaneous	70	2.68	10.1 ± 2.3
	Oral	50	7.5	6.5
Sodium sulphapyridine	Intravenous	70	0.96	9.0 ± 3.0
	Subcutaneous	70	1.39	28.7 ± 5.6
	Oral	60	2.7	10.0 —
Soluthiazole	Intravenous	60	1.28	14.4 ± 3.4
	Subcutaneous	50	2.57	12.5 ± 2.6
	Oral	50	8.0	15.2 —
Sodium sulphathiazole	Intravenous	80	0.99	12.3 ± 2.3
	Subcutaneous	70	1.81	5.8 ± 1.7
	Oral	65	4.5	5.5 —

dum's (4) method. These figures, which are in terms of the parent compounds, showed that solupyrindine was of the same toxicity as soluthiazole by all methods of administration, and also that the sodium salt of sulphapyridine was slightly more toxic than the sodium salt of sulphathiazole. The new compounds were both less toxic than the corresponding sodium salts.

The acute toxic symptoms in mice were as follows: With solupyrindine, symp-

toms appeared within five minutes after intravenous injection, characterised initially by trembling and rigidity of the tail. Ataxia and severe convulsions followed which were mainly of the clonic type. These continued intermittently for five or ten minutes, death resulting from respiratory failure after a particularly violent clonic convulsion. With soluthiazole, toxic symptoms appeared somewhat later, and severe convulsions of a mixed type were observed. The symptoms were similar to those caused by the sodium salts of the parent compounds.

Urolithiasis. We examined the compounds for the production of urolithiasis, although it is recognised that this toxic manifestation is a function of the solubility. An attempt was made to produce urolithiasis in rats by the administration of large doses of soluthiazole, and to compare the effect of equivalent doses of the parent substance. The amounts used were 10, 5 and 2.5 mg./g. per day. Soluthiazole was given subcutaneously in two doses each day, and sulphathiazole orally in suspension once daily. The majority of the animals given sulphathiazole had crystals in the kidneys and ureters at autopsy, whereas the animals given soluthiazole were entirely free from symptoms.

TABLE 5

The effect of solupyrindine and soluthiazole on phagocytosis

Figures indicate number of bacteria per phagocyte

	DRUG CONCENTRATION								TOXIC END-POINT
	0.8%	0.7%	0.6%	0.5%	0.4%	0.3%	0.2%	0.1%	%
Solupyrindine	0.8	3.4	8.8	9.6	21.1				0.8
Soluthiazole.				0.7	1.2	5.0	12.2	16.9	0.5

Local toxicity. (i) *Action on phagocytes.* We examined the technique of Thrower and Valentine (5) and evolved a simple method based on this technique without the use of reconstituted blood. The method is briefly as follows: Human citrated blood, freshly withdrawn, is mixed with dilutions of the compound and incubated at 37°C. for three hours. The antigen consists of an agar slope culture of *Staphylococcus aureus*, washed off into sterile saline, standardised to 1,000 million organisms per cc., and heated at 60°C. for one hour. This is added, and the blood is then reincubated for half-an-hour. The phagocytes are concentrated by lightly centrifuging in sealed capillary tubes; films are prepared stained by Gram's method, and the number of bacteria in each 25 phagocytes is counted. Readings of one coccus or less per phagocyte were taken as demonstrating inhibitory action, and this concentration was recorded as the toxic end-point. Typical results are shown in table 5.

These results indicated that neither soluthiazole nor solupyrindine was very toxic to leucocytes. Solupyrindine appeared slightly less harmful in this respect than soluthiazole.

(ii) *Effect on wounds.* The effect on wounds was tested by a method described

by Ungar and Robinson (6). The wounds are produced under anaesthesia on both sides of the shaven flank of guinea pigs by burning the skin with an electric iron for a period of five seconds. After five days the scabs are removed, and the wounds are then treated with the compound. We used 5 per cent concentrations, and the solutions were applied daily for a period of five days. Inspection of the wounds and microscopical examination of sections of skin tissue showed no significant difference in the rate of healing of treated and control animals.

(iii) *Action on the conjunctiva.* Repeated instillation of the undiluted 20 per cent solution of both compounds into the conjunctival sac of rabbits or guinea pigs produced no visible damage.

(iv) *Effect on skin tissue.* The irritant action was tested by a method described by Paget, Trevan and Attwood (7). Graded concentrations from 1 to 20 per cent were injected intracutaneously in quantities of 0.05 cc. into the shaven flank of guinea pigs. The lowest concentration which produced a clear local reaction during the period of observation (three days) was recorded as the minimal necrosing concentration. We found that neither compound produced a reaction in concentrations of 1 and 2 per cent; 5 per cent produced a very slight reaction, whereas with 10 and 20 per cent concentrations there was a definite indurated necrotic area, and even sloughing of the skin with the 20 per cent solution.

Discussion. In evaluating the activity of a drug in experimental infections, it is important to distinguish between the mere detection of activity and a quantitative estimate. This has been examined by Marshall, Litchfield and White (2). They based an estimate of the activity on the relationship between blood concentrations rather than on the actual doses given. They were, however, concerned mainly with compounds given orally, and therefore differences in the blood concentrations were obviously important. By relating the median survival dose to the blood concentration a measure of the activity was obtained, and in this way they found that in streptococcal and pneumococcal infections, sulphathiazole and sulphapyridine administered orally were of the same order of activity.

Although our own experiments with streptococcal and staphylococcal infections readily lend themselves to such an evaluation, for the purpose of presenting our results here we have adopted the simpler, although not necessarily less accurate, method described. Furthermore, blood absorption curves for soluthiazole and solupyridine, given in the following paper, were found to be almost identical, and since the substances were given by injection, absorption was more regular. Inspection of figs. 1 and 2 shows that as the responses were approximately parallel, an estimate could be made from the standard effective doses.

Soluthiazole and solupyridine were equally active. No significant difference in activity could be demonstrated against staphylococcal infections, although this is contrary to clinical findings, sulphathiazole having been reported more active than sulphapyridine. Our experiments were, however, carried out with only one strain of staphylococcus. Against streptococcal infections, the analogues were equal in activity to the sodium salts. The sodium salts were, however, more active in staphylococcal infections, and we are unable to account for

this since the evidence would appear to show that all the available sulphonamide in the analogues is liberated in the body.

An accurate estimate of the acute toxicity in mice showed that both by intravenous and by subcutaneous administration the analogues were less toxic than the corresponding sodium salts. The differences were not, however, very great, and if the toxicity were an indication of the active material present, it would provide confirmation that the analogues are not dissimilar from the sodium salts in the availability of the sulphonamide present. Presumably the cinnamylidene dibisulphite part of the molecule has little activity or toxicity. The anticipation that these neutral soluble derivatives would be better tolerated locally than the alkaline sodium salts was borne out by our findings.

SUMMARY

1. An examination has been made of the therapeutic activity and toxicity of soluthiazole and solupyrindine, disodium cinnamylidene dibisulphite derivatives of the parent compounds, sulphathiazole and sulphapyridine.

2. They were equally active, and in streptococcal and pneumococcal (Type I) infections in mice were similar in activity to the sodium salts of the parent compounds, but against staphylococci they were less effective.

3. Solupyrindine and soluthiazole were equal in toxicity to mice by all methods of administration, and were less toxic than the corresponding sodium salts of the parent compounds; they did not produce urolithiasis to any extent.

4. Tests for local toxicity and irritant properties indicated that the solutions were better tolerated than those of the sodium salts.

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THE ABSORPTION AND EXCRETION OF SOLUTHIAZOLE AND SOLUPYRIDINE

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Soluthiazole and solupyrindine are the disodium cinnamylidene bisulphites of sulphathiazole and sulphapyridine respectively. The absorption and excretion have been studied in various laboratory animals, and we have extended the investigation to field trials in sheep and cattle and also some observations in man. Both soluthiazole and solupyrindine were used in solutions containing the equivalent of 20 per cent of the parent substances, and all figures given are in terms of the parent compounds. The method of estimation was that described by Bratton and Marshall (1), using a photo-electric absorptiometer and 0.2 cc. of blood at a dilution of 1:50. Recovery from blood was good, presumably as the result of hydrolysis of the analogues to the parent compounds, but was not as regular as that obtained with the parent substances, although the error was not greater than ± 0.5 –1 mg. per cent. It is conceivable that the body fluids of animals receiving these drugs may contain (a) free sulphathiazole, (b) unchanged soluthiazole, (c) acetylated or otherwise conjugated sulphathiazole. Under the conditions of the procedure used for determination, we have been unable to distinguish (a) from (b), and thus our figures for free sulphonamide do not indicate the extent of any hydrolysis of soluthiazole which might take place in the body.

ABSORPTION INTO BLOOD-STREAM. *Mice.* Groups of mice (35 on each dose) were given graded doses, and at intervals groups of five mice were killed and blood concentrations determined. The results obtained with soluthiazole are shown in figs. 1 and 2, similar responses being obtained with solupyrindine. By all methods of administration the two compounds were absorbed rapidly, and even more quickly than the sodium salts. After intravenous injection, a peak concentration was reached within twenty minutes, and after subcutaneous or oral administration within thirty minutes. Although there was a rapid disappearance from the blood, it was possible by repeated subcutaneous injections at intervals of $1\frac{1}{2}$ hours to maintain an adequate concentration.

Absorption in other animals. These results are summarised in table 1.

The rate of absorption in the rabbit was similar to that in mice, and there was no marked difference between the rates of absorption of soluthiazole and solupyrindine, or between intramuscular and subcutaneous injection. This was due presumably to the high solubility of the compounds. The blood level could be maintained at 15 mg per 100 cc. with an initial subcutaneous injection of 0.125 g./kg, followed by 0.075 g./kg. at $1\frac{1}{2}$ -hour intervals. After the last injection, however, the blood level quickly fell (within four hours) to 5 mg. per 100 cc.

The blood concentration in the cat was maintained for a longer time. This

may have been due to the anaesthetic employed to allow for the withdrawal of cerebrospinal fluid by cisternal puncture. The peak concentration in the dog

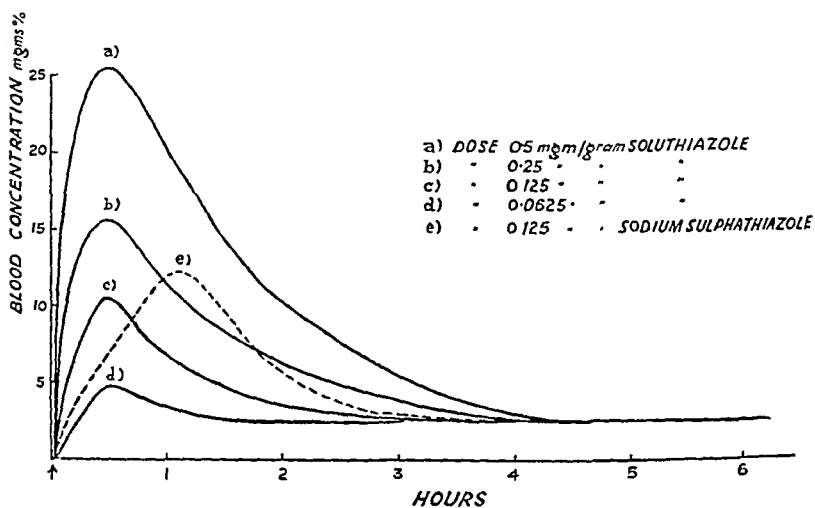


FIG. 1. BLOOD CONCENTRATIONS IN MICE AFTER SINGLE SUBCUTANEOUS INJECTIONS OF SOLUTHIAZOLE

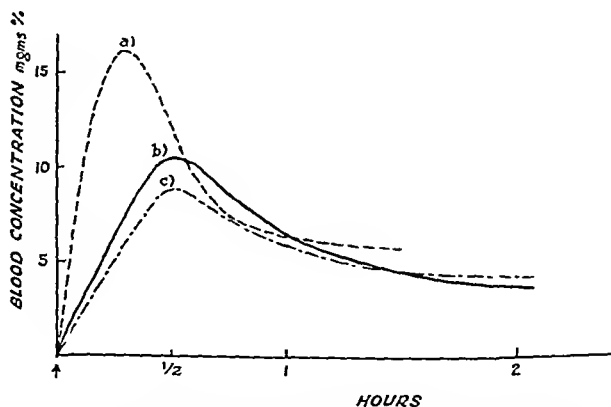


FIG. 2. COMPARATIVE RATE OF ABSORPTION OF SOLUTHIAZOLE IN MICE AFTER a) INTRAVENOUS, b) SUBCUTANEOUS, AND c) ORAL ADMINISTRATION
Dose: 0.125 mg./g.

occurred within half-an-hour, but in sheep and cattle it was somewhat later, and was maintained for a longer time. Absorption into the blood took place readily after infusion into the udders of cows.

TABLE 1

Concentrations of free sulphonamide in the body fluids of different animals

(a) Rabbit: 0.25 g./kg. solupyrindine subcutaneously

	HOURS				
	1	2	5	24	48
Blood concn., mg. per 100 cc.....	16.0	10.7	4.6	1.0	
C.S.F. concn., mg. per 100 cc.....	1.0	1.1	1.5		
Urinary excretion (% of dose).....		7.1	20.5	30.7	63.7

0.125 g./kg. soluthiazole intramuscularly

Blood concn., mg. per 100 cc.....	8.7	5.5	3.0	1.7	
Urinary excretion (% of dose).....		8.2	15.3	39.1	60.5

(b) Cat: 0.15 g./kg. solupyrindine subcutaneously

	HOURS				
	1	2	3½	5	7
Blood concn., mg. per 100 cc.....	7.8	7.3	7.1	6.3	
C.S.F. concn., mg. per 100 cc.....	1.1	1.0		2.5	

0.25 g./kg. soluthiazole subcutaneously

Blood concn., mg. per 100 cc.	8.6	15.8	15.1		11.7
C.S.F. concn., mg. per 100 cc.....	0.6	0.9	1.2		1.6

(c) Dog: 0.2 g./kg. solupyrindine intramuscularly

	HOURS				
	½	1	1	2½	5
Blood concn., mg. per 100 cc.... .	9.3	12.3	11.8	9.0	4.6

(d) Sheep: 0.35 g./kg. solupyrindine subcutaneously

	HOURS				
	½	1	3	5	7
Blood concn., mg. per 100 cc.. . . .	7.0	10.3	16.2	14.0	9.2

0.4 g./kg. soluthiazole subcutaneously

Blood concn., mg. per 100 cc.	11.6	12.8	18.2	16.6	16.4
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(e) Cow: 0.1 g./kg. solupyrindine subcutaneously

	HOURS				
	½	1	3	5	7
Blood concn., mg. per 100 cc.	1.9	2.4	4.4	3.9	2.8

Udder infusion, 225 cc. (4.5% soln.) into each quarter

Blood concn., mg. per 100 cc.. . . .		14.0	9.2	4.5	4.2
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Absorption in man. We have had an opportunity of studying the absorption in man, and these results are shown in table 2 and fig. 3.

TABLE 2
Blood concentrations in man

PATIENT NO.	BLOOD CONCENTRATION, MG. PER 100 CC.							
	½ hr.		1½ hrs.		3 hrs.		6 hrs.	
	Free	Total	Free	Total	Free	Total	Free	Total
(a) 5 cc. soluthiazole intramuscularly								
1	12.8	12.8	4.1	4.1	4.5	4.6	2.1	2.2
2	2.4	2.5	5.2	5.2	3.0	3.0	1.9	1.9
3	2.0	2.0	4.2	4.2	3.9	3.9	3.2	3.2
4	1.9	1.9	2.2	2.3	2.3	2.3	2.0	2.0
5	3.2	4.0	3.4	4.7	1.8	2.0	1.3	1.6
6	4.2	4.3	4.3	4.4	3.7	3.7	3.4	3.6
7	3.8	3.9	4.0	4.1	3.1	3.1	2.0	2.0
8	3.0	3.0	3.1	3.1	2.3	2.3	2.0	2.0
9	4.0	4.0	3.8	3.8	3.3	3.4	2.3	2.3
16	4.3	4.4	5.8	5.8	5.0	5.0	5.0	5.0
17	5.9	5.9	6.0	6.0	5.8	6.1	4.5	4.8
Average...	4.3	4.5	4.2	4.4	3.5	3.6	2.7	2.8
(b) 20 cc. soluthiazole intravenously								
11	11.7	11.7	9.5	9.5	8.1	8.1	5.9	5.9
12	14.4	14.4	12.0	12.0	8.0	8.8	6.2	6.7
13	19.0	19.0	14.2	14.2	12.0	12.0	7.6	7.6
14	13.5	15.3	11.0	12.4	7.6	9.1	5.2	6.1
15	13.9	15.6	9.1	11.8	7.6	7.9	5.2	5.8
Average...	14.5	15.2	11.2	12.0	8.7	9.2	6.0	6.4
(c) 20 cc. solupyrindine intravenously								
18	12.9	13.3	8.7	9.6	5.1	5.8	3.2	3.6
19	15.9	17.0	6.9	7.8	7.4	7.5	3.6	4.0
20	18.5	18.5	10.8	11.1	8.4	9.0	5.3	5.6
21	22.4	22.8	13.3	13.7	10.7	10.7	7.5	7.5
22	19.1	19.4	11.1	11.2	8.4	8.7	5.7	5.9
Average...	17.8	18.2	10.2	10.7	8.0	8.3	5.1	5.3

Patients 1 to 4 were civilian; the remainder were military, V.D. patients.

Although the series was small, absorption was satisfactory, and fairly regular with both compounds. The maximum level after intravenous injection occurred within half-an-hour, and this agreed with the results obtained in animals. The blood level quickly fell, but a dose of 20 cc. ensured an immediate high concentra-

tion which could then be maintained either by further injections, not more than six hours later, or by oral administration of the parent substance. Almost the whole amount present in the blood was in the free form; very little was conjugated, and there was no difference in this respect between soluthiazole and solupyrindine. This was of considerable interest, since it is commonly reported that with sulphapyridine amounts between 15 and 75 per cent are acetylated in the blood, and between 0 and 30 per cent for sulphathiazole. Conjugation of the sodium salts is, however, also very much less, being comparable with that of solupyrindine and soluthiazole, and presumably on account of the high solubility and rapid excretion the liver has little time in which to acetylate large amounts.

PENETRATION INTO THE CEREBROSPINAL FLUID, AND DISTRIBUTION IN THE TISSUES. We determined the concentration in samples of cisternal fluid, and found

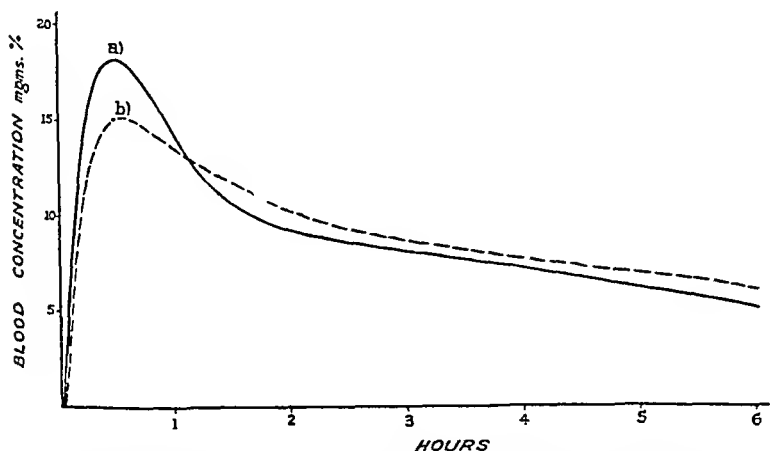


FIG. 3. BLOOD CONCENTRATIONS IN MAN AFTER A SINGLE DOSE OF 20 CC.

(a) Solupyrindine, given intravenously. (b) Soluthiazole, given intravenously. Average of 5 patients on each.

only small amounts. Results obtained in the rabbit and cat are shown in table 1. The low concentration may have been due to the anaesthetic employed in the procedure, but a probable explanation is the rapid excretion of these compounds. This was investigated further by examination of the distribution in the tissues of anaesthetised cats with ligated kidneys by a method described by Fisher, Troast, Waterhouse and Shannon (2). The compounds were administered intravenously, and at the conclusion of the experiment (two hours) blood samples were obtained and centrifuged immediately to separate cells from plasma. Cerebrospinal fluid was obtained by cisternal puncture, and samples of tissue were macerated with sand in 5 per cent sodium carbonate, the protein coagulated by heat, and the amount of drug in the filtrate determined. Calculations were based on mg./100 g. wet weight. The results are shown in table 3.

These experiments confirmed that penetration into the cerebrospinal fluid was slight. The largest amounts were accumulated in the liver and plasma.

EXCRETION AND CONJUGATION IN THE URINE. *Excretion in rats and rabbits.* On account of their high solubility the compounds were rapidly eliminated from the body. After only three hours 20 to 30 per cent, and within 24 to 48 hours more than 50 per cent, was excreted. Because of the rapid rate of elimination, the amount conjugated was less than would have been expected with the parent compounds given orally. These results are shown in table 4.

In order to discover whether the compounds were intrinsically less prone to conjugation, we carried out a comparative experiment in rats, giving the sub-

TABLE 3
Distribution of free sulphonamide in the tissues of the cat

COMPOUND	DOSE mg./g.	CONCENTRATION (mc./100 g.)					
		Plasma	R.B.C.	Muscle	Liver	C.S.F.	Brain
Soluthiazole.....	400	105	32	12.1	142	3.9	3.1
Solupyrindine.....	400	122	21	8.1	109	4.7	4.9

TABLE 4
The rate of excretion and conjugation in the urine after 0.25 mg./g. subcutaneously

ANIMAL	COMPOUND	PERCENTAGE DOSE EXCRETED		
		PERCENTAGE DOSE EXCRETED IN CONJUGATED FORM		
		8 hrs.	24 hrs.	48 hrs.
Rat	Soluthiazole	33.6	54.4	67.1
		2.4	3.1	4.0
Rabbit	Soluthiazole	15.3	39.1	49.5
		1.8	9.3	12.3
	Solupyrindine	20.5	30.6	63.7
		1.9	2.3	18.3

stances orally and comparing the amounts conjugated with those for the parent compounds, but found no significant difference. When given either by intramuscular or by subcutaneous injection, there was no difference between soluthiazole and solupyrindine in the rate of excretion or in the amounts conjugated; the amount conjugated was, however, definitely less than after oral administration of the parent compounds.

Excretory end-product. Soluthiazole and solupyrindine as such are highly soluble in urine and almost insoluble in organic solvents. For the purpose of ascertaining in what form these compounds were excreted, the urine of rabbits which had been given large doses was subjected to prolonged extraction with

ether, and in the extract some of the parent compound was isolated and identified, so that at least a proportion of the soluble derivative was excreted in the form of its parent substance. We have been unable to identify solupyrindine and soluthiazole as such in the urine.

Excretion in man. The rate of excretion and amounts conjugated have been studied in twelve patients, and the results are shown in table 5. A high proportion was eliminated within a very short time, and only a small amount was conjugated. Within 12 hours from 40 to 70 per cent was excreted, and the concentrations in the urine varied from 240 to 1,170 mg. per 100 cc.

DISCUSSION. The absorption and excretion of the sulphonamides depend on a number of factors; amongst these are the solubility of the compounds, the ease with which they are acetylated in the liver, and the solubility of the acetylated

TABLE 5
Urinary excretion in man
a = Percentage of dose excreted
b = Percentage of dose conjugated

TREATMENT	PATIENT NO.	1½ HOURS		3 HOURS		5 HOURS		12 HOURS	
		a	b	a	b	a	b	a	b
5 cc. soluthiazole intra-muscularly	16	19.9	1.93	31.9	3.15	51.7	5.13	75.4	9.73
	17	10.2	1.40	20.2	2.72	40.4	6.62	77.8	8.40
20 cc. soluthiazole intravenously	11	13.2	0.32	20.6	0.70				
	12	19.4	0.68	31.4	0.87				
	13	29.3	1.49	53.8	1.49				
	14	13.5	0.91	16.7	1.04				
	15	8.2	0.19	21.7	0.19				
20 cc. solupyrindine intravenously	18	11.0	2.07	16.1	4.17	29.5	5.75	42.9	7.71
	19	21.7	0.0	30.7	0.24	39.7	0.47	44.2	1.06
	20	28.8	2.40	39.5	2.40	50.5	3.76	55.2	4.7
	21	14.8	0.71	25.3	1.18	36.1	1.81	42.2	2.12
	22	20.0	2.24	28.4	4.66	35.6	7.08	40.7	8.25

compounds in the urine. A direct relationship between the amount conjugated in the blood and that in the urine does not always hold, possibly because of reabsorption of some of the free form from the tubules of the kidney back into the blood stream. The solubility in the urine and pH of the urine are important, since some acetylated sulphonamides are more soluble in alkaline urine than in water.

When we consider the relative absorption and excretion of the parent compounds, we find that sulphathiazole is more soluble than sulphapyridine, and therefore its speed of absorption and excretion is stated to be more rapid (Spink, (3)). It is also less prone to acetylation, probably because it remains in the bloodstream for a shorter time and there is less opportunity for acetylation in the liver to occur. With these new soluble derivatives, we found that the difference

between sulphathiazole and sulphapyridine in the amount conjugated largely disappears. They are both quickly absorbed into the blood, and the amounts conjugated are extremely small, considerably less than with the parent compounds. This was true both in animals and in man. There was no marked difference between the two compounds, although differences might become apparent if a larger series of cases was examined.

SUMMARY

The absorption and excretion of soluthiazole and solupyridine have been studied in animals and in man.

When injected intravenously, subcutaneously or intramuscularly, they provide a means of rapidly obtaining a high concentration in the blood, and the peak concentration occurred usually within half-an-hour. They are present in the blood almost entirely in the free form, very little being conjugated. There was little difference between the rates of absorption of soluthiazole and solupyridine either in animals or in man. The concentration in the cerebrospinal fluid was much lower than in blood.

Excretion in the urine was rapid, and in the series of human cases examined from 40 to 75 per cent of the dose was excreted within 12 hours. The amounts conjugated were less than 10 per cent.

We are indebted to Dr. A. H. Harkness, St. Charles' Hospital, Captain Twohig, R.A.M.C., Miss M. B. Cooper, M.R.C.V.S. and Miss Weddell, M.R.C.V.S., for obtaining samples for examination.

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SOLUTHIAZOLE AND SOLUPYRIDINE IN THE TREATMENT OF EXPERIMENTAL PYOCYANEAL INFECTIONS OF THE CORNEA

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Since soluthiazole and solupyrindine are highly soluble and form almost neutral solutions, we thought they should lend themselves to the local treatment of eye infections. Their biological properties have been described in a previous communication; they have advantages over the sodium salts since, while no less active in streptococcal infections, they are better tolerated by local application and are even more readily absorbed. The application of the sulphonamides in the treatment of eye infections has been thoroughly studied by Robson and Scott (1), who stated that suitable drugs should ideally comply with the following desiderata: high solubility, non-irritation, neutral solution and ready penetration.

EXPERIMENTAL METHODS. In the present communication we are concerned only with *Ps. pyocyanea* infections, although experiments are in progress with pneumococcal and staphylococcal infections, and these will be reported in due course. Although pyocyaneal ulcers are uncommon in man, they form experimentally in animals a ready means of evaluating the therapeutic effectiveness of a drug, and have been used in this way by Joy (2), v. Sallmann (3) and Robson and Scott (1). We followed the technique described by the last authors, with the exception that we used guinea pigs instead of rabbits. Having carried out experiments with rabbits we wished to widen the investigation by employing smaller animals. We found that the infection could readily and satisfactorily be produced in the eyes of guinea pigs. The eye of the guinea pig is probably not more different from that of man than is the eye of the rabbit.

The ulcers were produced by the removal, under anaesthesia, of about 2 x 2 mm. of the corneal epithelium and infecting this area with a platinum loopful of a fresh 18-hour agar slope culture. The soluble sulphonamides were applied initially as 20 per cent solutions (of the parent compound); these were then diluted as required to 10, 5 and 2.5 per cent solutions. Commencing one hour after infection, the solutions were applied as drops instilled into the conjunctival sac and onto the cornea every two hours, four times daily, for three days. The right eye was treated, the left eye being used as a control receiving normal saline only. The course of the infection was very similar in the guinea pig to that described by Robson and Scott (1942) for the rabbit. The lesions, however, were not permanent and we usually observed spontaneous healing after the first week.

RESULTS. I. *Effect of soluthiazole, 20°C., solupyrindine 20°C. and sodium sulphacetamide 30°C.* Soluthiazole 20°C.: At 24 hours there was little difference, but after a further 24 hours the ulceration was greater in the controls than in the treated eyes. Four days after discontinuing the applications the treated eyes appeared quite clean and free from any scar, while the untreated eyes showed ulceration and opacities.

Solupyridine 20%: The effect was similar to that for soluthiazole. Four days after treatment the eyes appeared normal, while the controls showed small ulcers and opacities.

Sodium sulphacetamide 30%: This treatment was similarly effective.

II. *Effect of soluthiazole 10% and solupyridine 10%*. After 24 hours all animals showed ulcers and small opacities of varying densities. When the drops had been discontinued for three days the treated eyes all showed improvement, while the controls showed severe ulceration. Three days later the treated eyes had almost healed (fig. 1). There was no difference in the effects produced by the two compounds, and they were as effective in a 10% as in a 20% concentration.

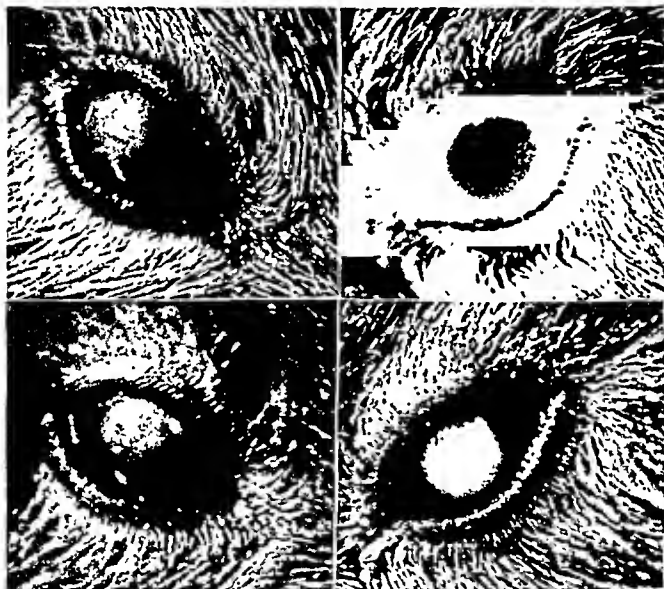


FIG. 1 PYOCYANEAL CORNEAL ULCERS IN GUINEA PIGS

Top: right, control infected eye after 6 days; left, treatment with soluthiazole (10%).
Bottom: right, control infected eye after 6 days; left, treatment with solupyridine (10%).

III. *Effect of soluthiazole and solupyridine, 10%, 5% and 2.5%*. Since we could not discern any marked difference between the effects of soluthiazole and solupyridine, descriptions given of the various treatments apply to both compounds.

Soluthiazole or solupyridine 10%: 24 hours after treatment there was greater ulceration in the control than in the treated eyes. At 48 hours the ulceration and opacity in the treated eyes were only slight compared with the controls. At six days slight ulceration was still present, but at eight days the ulcers had healed leaving only a slight corneal opacity.

Soluthiazole or solupyridine 5%: At 24 hours there was no difference. At 48

hours the ulceration was smaller in the treated eyes. Three days after treatment the same difference was noticeable, and throughout the ulceration was slightly less than in the controls.

Soluthiazole or solupyrindine 2.5%: This treatment was ineffective.

DISCUSSION. We have demonstrated that soluthiazole and solupyrindine are effective in pyocyaneal infections of the cornea. This is due presumably to the application of a high concentration with ready penetration, and is achieved without the irritation caused by solutions of the sodium salts. Repeated applications of 10 and 20 per cent solutions to uninfected eyes caused no damage to the conjunctiva or corneal tissues. It does not necessarily follow that the human eye would react in the same way, and in some preliminary clinical trials it has been found that the 10 per cent solutions are tolerated in the eyes of newborn infants, while in some cases the 20 per cent solutions cause irritation. This is, however, probably enhanced by the absence of tears from infants' eyes, and concentrations greater than 10 per cent might be tolerated by the adult eye. In any case, as far as experimental pyocyaneal infections are concerned, a concentration of 10 per cent (of the parent compound) is quite effective. We were interested in the fact that *Ps. pyocyanea* both in *in vitro* experiments and in wounds other than those of the eye shows little response to sulphonamide drugs, and the reason which appears to us most probable for the activity of these compounds against pyocyaneal infections of the eye is that there is little accumulation of pus. It remains to be determined how active these compounds are in other infections of the eye. We note that Robson and Scott (1) have tried another soluble derivative, sulphathiazole sodium formaldehyde sulphonylate, in staphylococcal infections, surprisingly without effect. There appears, nevertheless, room for the clinical use of neutral soluble sulphonamides, and Dickson (4) has already found that the institution of 10 per cent sodium sulphacetamide as a first aid measure produced a marked reduction in the incidence of corneal ulcers in miners.

SUMMARY

Soluthiazole and solupyrindine are effective against pyocyaneal corneal ulcers in guinea pigs. The minimal effective concentration is 5 to 10 per cent (of the parent compound), which is non-irritant.

It is a pleasure to record our appreciation to Dr. H. Wyers and Dr. R. J. Eadie for their help in some experiments, and to Mr. W. F. H. Rawles for the photographs.

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FURTHER STUDIES ON THE DETOXICATION OF THE ARSPHENAMINES BY ASCORBIC ACID

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In a previous study of the detoxication of neoarsphenamine by means of organic acids (1) it was suggested that the organic acid (i.e., usually ascorbic acid) may act a) by preventing or retarding the formation from neoarsphenamine of the much more toxic arsenoxide, or b) by peptizing colloidal neoarsphenamine. Rodman and Wright (2) have shown that the colloidal fractions of neoarsphenamine are about ten times as toxic as the whole drug. However, they have also shown that the amount of colloidal material present in the usual commercial sample is quite small, and its removal or peptization would cause a decrease in toxicity of only 10 to 15 per cent, while ascorbic acid is capable of decreasing the toxicity by about 50 per cent (1). A third possibility is suggested by the work of Probey, et al. (3). They have pointed out that only a part of the toxicity of neoarsphenamine is inherent in its arsenic content, and the remainder is due to the presence of the o-aminophenol groups. In other words, the possibility must also be considered that ascorbic acid acts by protecting these groups from oxidation.

The answer to these questions is of some importance since, if ascorbic acid does act by interfering with the conversion of neoarsphenamine to arsenoxide, it might also diminish the effectiveness of the drug. On the other hand, ascorbic acid might alter the rate, or to some extent the site, at which arsenoxide is formed without affecting the total amount eventually formed, or the efficiency of the drug. Such reports as are available (1, 4, 5) indicate that ascorbic acid does not decrease the therapeutic effectiveness of neoarsphenamine.

In the study of these problems, use has been made of both chemical and biological methods. The first concerns the chemical reactions of the drug, particularly its behavior on oxidation. The purpose has been to see whether ascorbic acid influences the rate of oxidation of neoarsphenamine by air, or alters the course of the reaction in any way. The biological approach has involved: a) a study of the effectiveness of ascorbic acid when given in different quantities, and at different times with respect to the arsenical, with the object of determining the probable site of the detoxication process (whether in the blood stream, or in the tissues where neoarsphenamine is temporarily stored), and b) a study of the effect of ascorbic acid on the retention of arsenic in the tissues.

The extreme susceptibility of neoarsphenamine to oxidation is well known. It is generally supposed that the path of oxidation, both *in vitro* and *in vivo* is as follows: neoarsphenamine \rightarrow arsenoxide \rightarrow arsonic acid \rightarrow arsenic acid + aromatic residue. It is reasonable to suppose that ascorbic acid, since it is a reducing agent, would retard the oxidative breakdown of neoarsphenamine to

arsenoxide, at least to some extent. However, the chemical reactivity of ascorbic acid, in the absence of the proper catalysts, is low (6), and, depending on the conditions, it might be entirely without effect on this process. The most direct approach to the problem would be to study the effect of ascorbic acid on solutions of neoarsphenamine undergoing oxidation by air. In order to do this it is necessary to be able to analyze solutions which contain any or all of the following: neoarsphenamine, arsenoxide, arsenoxide N-methanal sodium sulfonate, and arsonic, ascorbic, and dehydroascorbic acids. Rosenthal's method (7) has been generally used in the past for the determination of arsenoxide in arsenical solutions. His procedure has not seemed satisfactory for these experiments because the color reaction is given by whatever compounds in the mixture contain the o-aminophenol group, and also because the color reagent is reduced by ascorbic acid. Consequently, a different approach involving a new precipitating agent for neoarsphenamine has been used.

Chemical study of oxygenated neoarsphenamine solutions. Since several of the substances mentioned as possibly occurring in the mixtures to be analyzed can be distinguished only by means of their behavior toward oxidizing agents, it is necessary that at least one of them be removed quantitatively, or nearly so; otherwise the problem is too complex for solution. The reagent which has been used in this work for the precipitation of neoarsphenamine is the synthetic detergent, cetyl pyridinium chloride.¹ In dilute alcohol this reagent precipitates neoarsphenamine in a yellow granular form, and nearly quantitatively.

The analysis of the various mixtures described below has been carried out chiefly by means of iodine titrations. The reactions of the arsenicals with iodine have been studied previously (8, 9) but primarily as a means of determining their arsenic content: the reactions are nearly stoichiometric. The procedures and results of typical analyses are given below.

PROCEDURE. To 5 cc. of a 1.5% neoarsphenamine solution was added 5 cc. of 10% aqueous sodium acetate, 0.2 cc. of 1% starch solution, and 5 cc. of glacial acetic acid. The resulting solution was titrated with 0.1 N iodine until the addition of 2 or 3 drops gave a blue color which persisted for at least 5 seconds. For the sample used (arsenic content 20.0%, 750 gm. equals one mole) the titer was 10.4–10.6 cc., or an excess of 0.4–0.6 cc. over the theoretical value of 10.0. This end point is not entirely satisfactory, particularly in the case of aged solutions, as it fades rather rapidly, but in the case of the other arsenicals studied the values obtained are very close to the theoretical ($\pm 3\%$) and the end points are more stable. The values (as equivalents of iodine per mole) are: sulfarsphenamine, 8.0; arsphenamine, 8.0; Glyvarsenyl (3,4'-diacetyl-amino 4-hydroxyarseno-benzene 2' sodium glycolate), 8.0; phenarsine² (3-amino 4-hydroxyphenyl dichlorarsine hydrochloride), 2.0; and 3-amino 4-hydroxy phenylarsonic acid, 0.0. Under these conditions ascorbic acid requires two equivalents of iodine per mole, and dehydroascorbic, by definition, none.

Solutions of neoarsphenamine were not completely precipitated by cetyl pyridinium chloride, but the residue was sufficiently small to make the desired analyses possible. To

¹ Manufactured by the Edwal Laboratories, Chicago, Ill. The author is indebted to Mr. Morris Auerbach of the Winthrop Laboratories for having suggested the possibilities of this compound.

² Recently the names dichlorophenarsine and oxophenarsine are to some extent being substituted for phenarsine and arsenoxide, respectively.

10 cc. of a 1.5% solution of neoarsphenamine was added 10 cc. of a 4% solution of cetyl pyridinium chloride in 60% ethyl alcohol. After standing several minutes in a stoppered container, the solution was filtered through a No. 1 Whatman paper. The filtrate was, on some occasions, slightly cloudy, but could be clarified by returning to the filter several times. An aliquot of 10 cc. was taken for analysis; a few cc. of glacial acetic acid were added, then 0.1 N iodine was run in a few drops at a time until a yellow color, stable for at least 30 seconds, was obtained. (Note: An excess of iodine reacts with the excess of precipitating agent to give a yellow product, which thus serves as a satisfactory indicator.) The value obtained was a 0.50 cc. of 0.1 N iodine, or about 5% of the amount required for the neoarsphenamine originally taken. This value is due to a small residue of unprecipitated arsenical, since it is constant for the given conditions, providing the amount of neoarsphenamine taken for analysis does not exceed the combining power of the precipitant; in this particular case a two-fold excess of precipitating agent is used.

Arsenoxide N-methanal sodium sulfonate should also be precipitated under the conditions described above since its sodium ion would be replaced by the quaternary ammonium ion.¹ This compound is undoubtedly the one which would be formed during the oxidation of neoarsphenamine, rather than arsenoxide N-methanal sodium sulfoxylate, since the redox potential of the sulfoxylate group is less than that of the arsenobenzene linkage (see table 4). However, 3-amino, 4-hydroxy phenylarsonic acid is not precipitated under the given conditions.

Neoarsphenamine was separated from arsenoxide as follows: In a 100 cc. graduated cylinder was measured 1.00 gm. each of neoarsphenamine and phenarsine, 870 mgm. of sodium bicarbonate, and water to 100 cc. Of this solution, 5 cc. required 9.8 cc. of 0.1 N iodine when titrated in sodium acetate-acetic acid; theory 10.1 cc. The precipitation was now carried out with cetyl pyridinium chloride as described above. A 10 cc. aliquot of the filtrate required 4.2 cc. of 0.1 N iodine as against 3.45 cc. which would be required for the arsenoxide alone. The difference between the theoretical value and that actually obtained is nearly the same as the blank value found with neoarsphenamine alone (0.5 cc. for 10 cc. of the filtrate).

A mixture containing neoarsphenamine, arsenoxide, and ascorbic acid was analyzed in the following way: A stock solution was prepared which contained, per cc., 10 mg. each of neoarsphenamine, phenarsine, and ascorbic acid, with sufficient bicarbonate to bring the mixture to neutrality. Of this solution, 5 cc. when titrated with 0.1 N iodine in sodium acetate-acetic acid required 15.8 cc., the same as the theoretical value. (When a considerable amount of ascorbic acid is present the end points are more precise.) After precipitation with cetyl pyridinium chloride, 10 cc. of the filtrate required 9.95 cc. of 0.1 N iodine to the yellow end point. The theoretical value (combining power of the arsenoxide plus ascorbic acid) is 9.15 cc. Again, if one subtracts the usual blank value of 0.5 cc. for unprecipitated neoarsphenamine, the two figures are brought into substantial agreement.

The partition between arsenoxide and ascorbic acid may now be determined by an analysis of the filtrate for arsenic. (Note: In the experiments which were subsequently run the arsenic in the filtrate was likely to consist only of unprecipitated neoarsphenamine and arsenoxide, or at least of arsenic in the form $R-As = 0$, since only prolonged aeration will cause the formation of arsonic acid from arsenoxide (see table 3). It is further unlikely that any arsonic acid would be formed in the presence of unchanged neoarsphenamine.)

¹ In order to study this point a solution of the compound in question was prepared by dissolving 600 mgm. of sulfarsphenamine (arsenic content, 21.5%) in 25 cc. of water, adding 34.5 cc. of 0.1 N iodine, enough bicarbonate to bring the solution to neutrality, and water to 65 cc. Of this solution, 5 cc. should contain 41.3 mgm. of arsenoxide N-methanal sodium sulfonate. The iodine titration for 5 cc. was 5.55 cc. of 0.05 N; theory 5.30. After precipitation with cetyl pyridinium chloride the iodine titration for 5 cc. was 1.0 cc. of 0.05 N. This indicates that about 80% of the compound was precipitated, or that 8 mgm. remained in solution.

A 20 cc. portion of the filtrate, prepared from the solution described immediately above, was taken for analysis for arsenic. The alcohol was first boiled off; about 1 cc. of concentrated sulfuric acid was added, followed by 2.5 gm. of powdered potassium permanganate in small portions. From this point on the procedure was identical with that given in U.S.P. XII. The final titration was made on a 20 cc. aliquot with a microburette, using 0.1 N thiosulfate, and 7.2 cc. was required. The blank value for the reagents used in the arsenic determination was 0.4 cc., leaving 6.8 cc. for 20 cc., or 3.4 cc. for a 10 cc. portion of filtrate. Subtraction of the usual neoarsphenamine blank leaves 2.9 cc. for the arsenoxide in the filtrate. The theoretical value is 3.45 cc., indicating a recovery of 82%. This method of analysis is, therefore, not very exact, but is capable of revealing the presence of significant amounts of arsenoxide in neoarsphenamine filtrates, or an increase in the amount of arsenoxide on exposure to air.

TABLE 1

Results of exposure to air of solutions of neoarsphenamine with and without ascorbic acid

SOLUTION	TIME OF EXPOSURE	TOTAL IODINE EQUIVALENT*	IODINE EQUIVALENT OF ARSEN- OXIDE PLUS ASCORBIC ACID†	IODINE EQUIVALENT OF ARSENOXIDE‡	TOXICITY OF SOLUTION§ LD ₅₀ ± S.E.‡
	days				
A. 1 cc. = 15 mgm. neo-arsphenamine	0	10.3	0	0	418 ± 11
	1	10.0	0.70	0	
	2	8.5	0.90	0.15	
	3	7.0	0.80	0.20	155 ± 15
	4	6.0	0.80	0.40	
B. 1 cc. = 11.25 mgm. neoarsphenamine plus 4.4 mgm. ascorbic acid	0	10.5	2.40	0	600 ± 25
	1	10.1	2.90	0	
	2	8.7	3.00	0	
	3	7.5	2.70	0.10	190 ± 8
	4	6.9	2.80	0.25	

* All iodine equivalents are given in terms of cc. of 0.1 N solution required for a 5 cc. aliquot of the original solution or a 10 cc. aliquot of the filtrate.

† The neoarsphenamine blank value of 0.5 cc. for 10 cc. of filtrate has been subtracted in all cases.

‡ As mgm./kgm. of neoarsphenamine.

§ Standard error, estimated graphically (see Miller, L. C., and Tainter, M. L., Proc. Soc. Exp. Biol. and Med., 57: 261, 1944).

The effect of atmospheric oxygen on solutions containing neoarsphenamine and neoarsphenamine plus ascorbic acid was now studied by allowing the two solutions to stand in the dark, but open to the air. Aliquots were analyzed at daily intervals by the method already described. Solution A contained 750 mgm. of neoarsphenamine in 50 cc. of water. Solution B contained 562 mgm. of neoarsphenamine, 220 mgm. of ascorbic acid, and 158 mgm. of sodium bicarbonate in 50 cc. Solution B was thus made up so that the total combining power for iodine would be the same as for (A) but exactly one-fourth of the neoarsphenamine would be replaced by ascorbic acid. The results are given in table 1.

The data in table 1 may be interpreted as follows: The total oxygen uptake, as indicated by the decrease in iodine titration, is almost in direct proportion to the neoarsphenamine content. From this it would be inferred that the ascorbic acid probably undergoes no change. The constancy of the [ascorbic acid plus

arsenoxide] fraction of Solution B points to the same conclusion. The possibility cannot be entirely eliminated that the ascorbic acid undergoes some change since it is known (10) that dehydroascorbic acid is very unstable above pH 5 and changes over rapidly to a substance more strongly reducing than ascorbic acid itself, but it would be more logical to assume that there is no change.

The amount of arsenoxide formed in both solutions is surprisingly small, and does not account for the total decrease in iodine equivalent, but it could account for the increase in toxicity.⁴ Since both solutions darkened considerably, it seems evident that the oxidative change involved primarily the o-aminophenol groups.

The neoarsphenamine solution was, as would be expected, always more toxic than the neoarsphenamine with ascorbic acid. However, the *relative increase* in toxicity of both solutions with time was practically the same. The ascorbic acid may be considered as functioning in one of two ways: 1) by delaying chemical changes in the arsenical during the oxygenation, and, possibly more important, 2) by detoxifying *in vivo* the unchanged neoarsphenamine which remains after the oxygenation. The following experiment was set up to investigate this question.

Solution C was made up to contain 1500 mgm. of neoarsphenamine in 50 cc. of water. Solution D contained the same amount of neoarsphenamine plus 375 mgm. of ascorbic acid and 300 mgm. of sodium bicarbonate. The solutions were left open to the air at 37°C. for three days, then the same quantities of ascorbic acid and bicarbonate were added to solution C, after which both were diluted to 75 cc. for analysis. They were also tested for toxicity in the albino rat, with the results given in table 2.

The data demonstrate once more the fact that the amount of arsenoxide formed during the oxygenation of neoarsphenamine is surprisingly small and that the ascorbic acid, when present, seems to remain unchanged. The principal change of the solutions was a darkening, both finally absorbing about 8 times as much light as a fresh neoarsphenamine solution of the same concentration when measured in a photoelectric colorimeter with filter S54. The color change in solution D was somewhat delayed, but it finally had a deeper color. Solution D was, if anything, slightly more toxic than C, demonstrating that the presence of ascorbic acid during the time of exposure to air did not prevent the chemical changes which result in an increase in toxicity. Therefore, an aged neoarsphenamine-ascorbic acid solution is less toxic than an equally aged neoarsphenamine solution simply because it was less toxic at the outset.

It is difficult to say in the case of neoarsphenamine whether the increase in

⁴The LD₅₀ of neoarsphenamine (whole drug) is usually about 400 mgm./kgm., or 240 mgm./kgm. of pure drug since approximately 40% of NaCl is added to the commercial product. The LD₅₀ of arsenoxide is 14 mgm./kgm. (computed from phenarsine whose LD₅₀ is 20 mgm./kgm.,⁵ and the conversion factor of phenarsine to arsenoxide is 0.69). If it is assumed that the molar toxicity of arsenoxide and of the N-substituted compound is the same, the approximate toxicity at any stage of the breakdown of neoarsphenamine to arsenoxide may be calculated. An LD₅₀ of 155 mgm./kgm. corresponds approximately to a 12% breakdown. This is of the right order of magnitude for the amount of arsenoxide found, but the decrease in total iodine equivalent at this point would indicate a much more extensive breakdown.

toxicity on exposure to air is due to breakdown to arsenoxide, or to oxidation of o-aminophenol groups, or both, since the process of oxidation is evidently complex. Whether the oxidation of o-aminophenol groups actually results in an increase in toxicity is more clearly seen in the case of arsenoxide itself, a compound of relatively simple structure; an oxidation of the As^{+++} to As^{++++} is known to decrease the toxicity of the compound. A study of the behavior of arsenoxide on oxygenation was therefore carried out, both in the presence and absence of ascorbic acid.

Solution P contained 500 mgm. of phenarsine and 435 mgm. of sodium bicarbonate in 100 cc. Solution PA contained 500 mgm. of phenarsine, 300 mgm. of ascorbic acid, and

TABLE 2

Chemical analysis and toxicity of solutions of neoarsphenamine and of neoarsphenamine plus ascorbic acid, aged three days

SOLUTION	ORIGINAL COMPOSITION	FOR SOLUTION	IODINE TITER [†] FOR FILTRATE	FOR ARSENIC [‡]	TOXICITY	
					Dose, mgm./kgm. neoarsphenamine	Mortality
C	1500 mgm. neoarsphenamine in 75 cc.*	11.4	3.7	0.18	150	0/5
					225	3/5
					300	4/5
D	1500 mgm. neoarsphenamine, 375 mgm. of ascorbic acid and 300 mgm. bicarbonate in 75 cc.	10.0	3.0 [§]	0.30	150	0/5
					225	4/5
					300	6/6

* Same quantities of ascorbic acid and bicarbonate as in Solution D were added after aging, before the analyses were made.

[†] All figures given are as cc. of 0.1 N iodine required for 5 cc. of the solution, or 10 cc. of the filtrate after precipitation with cetylpyridinium chloride. Neoarsphenamine blank value of 0.5 cc. subtracted.

[‡] For 10 cc. of filtrate, using U.S.P. XII method, and subtracting 0.5 cc. blank for unprecipitated neoarsphenamine.

[§] Theoretical value for the ascorbic acid alone, if unchanged, 2.84 cc.

655 mgm. sodium bicarbonate in 100 cc. Both solutions were neutral to litmus. They were exposed to the air and samples were titrated at intervals with iodine in sodium acetate-acetic acid solution. The solutions were also tested at intervals for toxicity in the albino rat. The results are given in table 3.

This experiment demonstrates two significant points. In solution P two separate types of oxidation were going forward. There was oxidation of o-aminophenol groups, as evidenced by the darkening of the solution, and there was oxidation of As^{+++} to As^{++++} , as evidenced by the decrease in iodine titer. However, there was a latent period of two days in which no change took place in the valence of arsenic, then during the next three days there was a marked change. In solution PA there was also little change in titer during the first two days, but a rapid

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					300	6/6

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change from the third to the fifth day. In solution PA there was never much color change, indicating that the ascorbic acid was protecting the o-aminophenol groups from oxidation.

There was little difference in the toxicity of the two solutions when freshly prepared (see also footnote 4). During the first two days there was an increase in the toxicity of solution P, coinciding with the period during which oxidation of o-aminophenol groups was occurring. During the next three days there was a decrease in toxicity, coincident with the oxidation of the arsenic. However, it must be stated that the extent of change from As^{+++} to As^{++++} would not account quantitatively for the observed decrease in toxicity, in spite of the fact that arsonic acid is much less toxic than arsenoxide.

TABLE 3

The effect of exposure to air on solutions of phenarsine and of phenarsine plus ascorbic acid

COMPOSITION OF SOLUTION	TIME OF EXPOSURE	TOTAL IODINE EQUIVALENT*	TOXICITY $\text{LD}_{50} \pm \text{S.E.}^\dagger$
	days		
0.5% phenarsine	0	8.67†	19.3 \pm 1.60
	1	8.76	—
	2	8.66	15.3 \pm 0.71
	3	8.33	—
	5	7.60	20.5 \pm 1.15
0.5% phenarsine, 0.3% ascorbic acid	0	17.30‡	18.0 \pm 0.73
	1	16.80	—
	2	16.75	19.0 \pm 0.75
	3	16.20	—
	5	14.30	23.4 \pm 1.35

* As cc. of 0.1 N iodine required for 5 cc. of solution.

† Theory, 8.65 cc.

‡ Theory, 17.18 cc.

§ As mgm./kgm. of phenarsine + standard error (estimated graphically).

In solution PA there was no change in toxicity during the "latent" period of the first two days, but there was a significant decrease in toxicity during the third to fifth days. Here the change in the valence of the arsenic, as revealed by titration, is sufficient to account for the decrease in toxicity. (It is assumed that the ascorbic acid underwent no change; no reasonable interpretation of the results is possible otherwise.)

Discoloration of arsenical solutions as an indication of increased toxicity. Bundesen et al. (11) have shown that solutions of neoparsphenamine and Mapharsen can be prevented from darkening on exposure to air if sufficient ascorbic acid is present. In the present work it has also been found that 1% neutral solutions, or suspensions, of phenarsine, 3-amino 4-hydroxyphenylarsonic acid, arsphenamine, and o-aminophenol darken on exposure to air for a day or more. These same solutions do not darken even in the course of a week if an equal weight of sodium ascorbate is present. Solutions of sulfarsphenamine and Glyvarsenyl do not

darken noticeably either in the presence or absence of ascorbic acid. Ascorbic acid does not decrease appreciably the toxicity of phenarsine,⁵ or of sulfarsphenamine,⁶ but it does decrease the toxicity of neoarsphenamine, arsphenamine,⁷ and Glyvarsenyl (1). It is, therefore, apparent that the effect of sodium ascorbate in decreasing the toxicity of certain arsenicals is not necessarily related to its property of preventing them from darkening on exposure to air. In general, however, ascorbic acid decreases the toxicity of the preparations which are the most unstable toward oxidation and has little effect on the stable preparations. Glyvarsenyl and phenarsine are exceptions to this generalization. The latter exception may be explained on the basis that although phenarsine can and does undergo oxidation of the o-aminophenol group, more prolonged treatment is required to cause this oxidation to occur extensively than in the case of neoarsphenamine⁸ or arsphenamine. Furthermore, the oxidation of As^{+++} to As^{++++} which occurs at the same time decreases the toxicity. No explanation of the behavior of Glyvarsenyl is apparent at this time.

Oxidation-reduction potentials of significant systems of arsenical compounds. The relationship of ascorbic acid to the oxidation of the arsenical compounds in the blood stream may also be studied by means of oxidation-reduction potentials. The potentials of many systems important to this study have already been determined, but those of the various stages of the oxidation of the arsphenamines do not seem to have been reported. The procedures used were as follows:

Quantities of the two components of the system were taken so that each would contribute 25 mgm. of arsenic. These were dissolved in about 75 cc. of oxygen-free water, with the help of bicarbonate, if necessary. Concentrated HCl, 85 cc., was then added and the solution was diluted to 100 cc. This makes a solution containing an equal number of equivalents of the oxidized and reduced form, and normal in hydrogen-ions. In cases where precipitation occurred on acidification, the quantities of both components were reduced equally until a point was reached at which no precipitation occurred. The solution was poured into a beaker, covered at once with mineral oil, and its potential was read on a Beckman electrometer. The electrodes used for this purpose consisted of one gold-plated platinum electrode and a normal calomel electrode. There was usually a rather rapid drift to an equilibrium value, which was reached after one or two minutes. After correction

⁵The LD_{50} of phenarsine determined with groups of 10 rats to a dose was 20 mgm./kgm. The LD_{50} of phenarsine plus ascorbic acid determined with groups of 5 rats to a dose was as follows (with sodium ascorbate added in the molar ratios indicated): 1 mole per mole phenarsine, 21 mgm./kgm.; 3 moles per mole phenarsine, 22 mgm./kgm.; 6 moles per mole phenarsine, 26 mgm./kgm. Similarly, the LD_{50} of phenarsine with sodium isoascorbate added in the ratios of 1, 3, or 6 moles per mole of phenarsine was 18 mgm./kgm. It is not clear why the ascorbate and isoascorbate should not give identical results.

⁶The LD_{50} of the arsenical as determined with groups of 15-20 rats to a dose was 590 ± 20 mgm./kgm. With 120 mgm. ascorbic acid (as the sodium salt) added, the LD_{50} determined with groups of 10 rats to a dose was 625 ± 16 mgm./kgm.

⁷The LD_{50} of the arsenical as determined with groups of 15-20 rats to a dose was 190 ± 5 mgm./kgm. With 100 mgm. of ascorbic acid added (as the sodium salt), a dose of 200 mgm./kgm. killed 2/10 and the LD_{50} was greater than 240 mgm./kgm.

⁸The passage of a rapid current of air through a neutral solution of phenarsine for 2-3 hours causes only a slight discoloration, while neoarsphenamine undergoing the same treatment becomes quite dark.

for the potential of the calomel electrode (-0.248v.), the E_0 could be taken simply as the potential difference, since there were present an equal number of equivalents of the oxidized and reduced forms, in normal acid solution. The readings were made at room temperature (22°C.) and the results are given in table 4.

It is clear that the arsenicals would be most likely to oxidize while being transported in the blood stream. While they are stored in the liver or kidney they should remain unchanged. It does not appear from the data that the oxidation potential of blood is high enough to cause the oxidation of o-aminophenol groups. However, it has already been shown that even atmospheric oxygen is capable of causing this change to occur. The presence of an excess of ascorbic acid would be

TABLE 4

Oxidation-reduction potentials of some systems of interest in the arsenical series

SYSTEM	E_0	rH^*
Mammalian liver.....		2.4 (12)
Mammalian kidney.....		3.1-6.4 (12)
Neoarsphenamine- $\frac{1}{2}$ sulfarsphenamine†.....	+0.310 v.	10.3
Arsphenamine-arsenoxide.....	+0.378 v.	12.6
Neoarsphenamine + 4 equiv. H_2O_2 †.....	+0.380 v.	12.7
Sulfarsphenamine + 2 equiv. H_2O_2 †.....	+0.390 v.	13.0
Ascorbic acid-dehydroascorbic acid.....	+0.390 v. (13)	13.0
Glyvarsenyl + 2 equiv. H_2O_2 †.....	+0.428 v.	14.3
Arsenoxide-arsonic acid.....	+0.550 v.	19.3
Human blood (pH 7.4).....	$E'_0 = 0.200\text{ v.}$ (14)	21.5
o-Aminophenol-o-iminoquinone§.....	+0.790 v. (15)	26.3

$$*rH = \frac{2F}{RT} E_{\pi} + 2 \text{ pH.}$$

† I.e., this is the redox potential of the sulfoxylate group.

‡ The quantities of H_2O_2 added were intended to cause the oxidation of half of the arsenical to the arsenoxide stage.

§ This potential cannot be determined directly because the oxidation product exists only temporarily, if at all. However, the potential of the system p-aminophenol-p-iminoquinone has been measured and its "apparent oxidizing potential" is about the same as this system.

expected to decrease the normal rate of oxidation processes in the blood. It has been shown by Bembé and Dietrich (16) that the presence of an excess of ascorbic acid does, in fact, markedly decrease the oxidation-reduction potential of blood.

Biological investigation of the mechanism of ascorbic acid action. Sandground (17) has studied the mechanism of detoxication of pentavalent arsenicals by p-aminobenzoic acid by investigating the "time factor"; i.e., the change in the detoxifying effectiveness of the acid when it is injected at different time intervals with reference to the arsenical. He found that the acid is effective if injected up to three hours before the arsenical, but is markedly less effective if injected 30 minutes or more after the arsenical. Martin and Johnson (18) reported that ascorbic acid is most effective as a detoxicant for arsenicals if injected two hours

previously, in order to permit complete diffusion in the tissues. We (1) have not been able to confirm this.

In this study the hypothesis was adopted rather early that a) the effectiveness of ascorbic acid depends upon its being present at a high concentration in the blood while the arsenical remains in the circulation, and that b) the detoxication takes place in the blood, not in the tissues. In other words, the extent of the detoxication achieved is a function of the concentrations of ascorbic acid and arsenical measured at the first moment when both are present simultaneously in the blood stream. Support for this hypothesis was obtained in the experiments which follow.

TABLE 5

Effect of injecting neoarsphenamine and ascorbic acid (as the sodium salt), at different time intervals, on the mortality of albino rats

DOSE		TIME BETWEEN INJECTIONS	MORTALITY
Ascorbic acid	Neoarsphenamine		
A. Ascorbic acid followed by neoarsphenamine			
mgm./kgm.	mgm./kgm.	minutes	
0	425		7/10
100	425	0	0/8
100	425	10	3/10
100	425	30	6/10
150	425	30	9/15
200	425	30	2/11
300	425	30	0/5
B. Neoarsphenamine followed by ascorbic acid			
0	425		5/10
100	425	10	2/10
100	425	30	6/10
200	425	30	7/9
300	425	30	5/8
600	425	30	3/8
1000	425	30	4/7

All animals used as subjects in these experiments were albino rats from the laboratory colony, weighing from 80 to 120 gms., and from 35 to 50 days of age. They were fasted for 16 hours prior to the injections, which were made into the saphenous vein in the usual manner. Eight per cent solutions of neoarsphenamine and four per cent solutions of ascorbic acid (with enough sodium bicarbonate added to form the sodium salt) were injected. The time at which each animal received the first injection was noted, and, at exactly the time interval desired, the second injection was begun. Deaths observed within 6 days are recorded in table 5 (A).^{*}

The data show, in brief, that 100 mgm./kgm. of ascorbic acid, if given simultaneously, will protect all animals from a dose of neoarsphenamine which, if given

^{*}The author is indebted to Mr. Donald Seppelin, Miss Evelyn Bricc, and Mrs. Charles Miller for carrying out the toxicity studies.

alone, would have killed about 60 per cent. The mortality rate is significantly increased if the ascorbic acid is given as much as 10 minutes before or after the neoarsphenamine and, if the time interval between the injections is increased to 30 minutes, this amount of ascorbic acid confers no protection whatsoever. Complete protection (i.e., 100 per cent survival) can be obtained by giving ascorbic acid 30 minutes before the arsenical, providing the dose is raised to a level of between 200 and 300 mgm./kgm. However, if the ascorbic acid is given 30 minutes after the neoarsphenamine, even 1000 mgm./kgm. confers no protection.

The results of part (A) of this experiment can be explained in terms of the blood levels of ascorbic acid which are observed following massive injections, as previously published (1). Thus, the injection of 400 mgm./kgm. of ascorbic acid results in a temporary plasma level of 430 mgm. per cent which has, in 30 minutes, decreased to about 120 mgm. per cent. From this it can be computed roughly that a dose of 100 mgm./kgm. would result in an initial plasma level of 110 mgm. per cent, which would, after 30 minutes, decrease to between 30 and 40 mgm. per

TABLE 6

Effect of ascorbic acid on the retention of neoarsphenamine by tissues

GROUP	TIME AFTER INJECTION	DOSE		AVERAGE ARSENIC CONTENT OF TISSUES*	
		Neoarsphenamine	Ascorbic acid	Liver	Kidney
	hrs.	mgm./kgm.	kgm./kgm.		
A	24	200	0	4.72 \pm 0.27	9.20 \pm 0.64
B	24	200	50	5.26 \pm 0.26	11.90 \pm 0.50
C	24	300	75	6.34 \pm 0.16	14.88 \pm 0.75
D	48	200	0	2.86 \pm 0.22	6.74 \pm 0.37
E	48	200	50	2.60 \pm 0.28	5.20 \pm 0.42
F	48	300	75	2.62 \pm 0.34	7.56 \pm 1.34

* Expressed as mgm. of As_2O_3 per 100 gm. of fresh tissue \pm standard error of the mean.

cent. Therefore, if the extent of detoxication depends on the blood level of ascorbic acid, the injection of 100 mgm./kgm. of ascorbic acid 30 minutes before the arsenical should result in the same mortality as if 30 mgm./kgm. were injected simultaneously with the arsenical. The latter dose is not sufficient to confer any protection (1), and none results here. However, if 100 mgm./kgm. were injected only 10 minutes before the neoarsphenamine, the blood level would be materially higher than 30-40 mgm. per cent when the arsenical was injected and some protection would be expected and is found here.

On the other hand, it can be reasoned that when ascorbic acid is given after the arsenical its effectiveness should be even more radically decreased. Neoarsphenamine is known to disappear very rapidly from the blood stream (about 75 per cent is actually removed within the first two minutes); consequently, if ascorbic acid acts only in the blood stream, it can act upon only the portion which remains. It appears, in fact, that after the lapse of 30 minutes it would be impossible to give enough ascorbic acid to confer complete protection.

Still another biological approach to this problem has been suggested by the work of Hogan and Eagle (19). They have shown that the amount of arsenic remaining in liver and kidney 24 or 48 hours after the intravenous injection of various substituted arsenoxides or arsonic acids is proportional to their toxicity. The effect of ascorbic acid on the retention and distribution of the arsenic of neoarsphenamine was studied by McChesney et al. (1). Although there was noted a slight tendency toward lower kidney concentrations of arsenic when ascorbic acid was given, the difference was not great enough to be impressive. So far as liver was concerned, the ascorbic acid made no difference in the retention of arsenic. In view of the findings of Hogan and Eagle, it has seemed worth while to repeat these experiments, but using smaller doses in order that, as suggested by these authors, the tissues would not be so altered by the toxic effects of the drug as to result ultimately in the death of the animal. The animals were prepared for the experiment as previously described (1). The tissues which were removed were kept frozen until the digestions could be carried out. Arsenic analyses were carried out by the method of Levvy (20), and the results are given in table 6. It is again demonstrated that the ascorbic acid has not altered the retention or distribution of the arsenic significantly. If the principles stated by Hogan and Eagle applied in this case it would be expected, for example, that the tissue levels of arsenic in groups A and C would be the same, and those of group B would be less than either. (The dose of 300 mgm. of neoarsphenamine plus 75 mgm. of ascorbic acid is about equivalent in toxicity to that of 200 mgm. of neoarsphenamine alone.)

DISCUSSION OF RESULTS. It has been generally supposed that when solutions of neoarsphenamine are exposed to the air they gradually undergo oxidation to the arsenoxide form. This is based on the observations of Rosenthal (7) and of Schamberg, Kolmer, and Brown (21). Rosenthal did not report how arsenoxide N-methanal sulfoxylate would react in his procedure, as he did not have the compound available. He was of the opinion that the side chain would split off during the oxidation. As has been pointed out, there is nothing specific about the Rosenthal color reaction for arsenoxide since other compounds which contain the o-aminophenol group give the same reaction. The only characteristics of arsenoxide upon which an analytical method can be based are the o-aminophenol group, the trivalent arsenic, and the relatively high solubility throughout the pH range. The validity of any analytical method, therefore, depends largely upon the extent to which a physical separation of arsenoxide from the other probable components of the mixture can be made. The precipitating agent which has been used in this work removes all but a small constant fraction of unchanged neoarsphenamine, most of the arsenoxide N-methanal sulfonate (and, presumably any other compounds containing the N-methanal sulfoxylate or sulfonate radical, since these would combine with the cetyl pyridinium radical). The reagent does not precipitate arsenoxide or arsonic acid, but the latter could be distinguished on the basis that its iodine titration is zero.

The method used in this work indicates that during oxygenation of neoarsphenamine solutions the following changes occur: a) a rather rapid drop in the iodine

titer from 10 equivalents per mole to a value which eventually approaches 4 equivalents per mole (this taken by itself would indicate complete oxidation to the arsenoxide stage since 2 equivalents per mole are required to oxidize the sulfoxylate group to sulfonate, and 4 equivalents to oxidize the arsenobenzene linkage to arsenoxide), b) a change in color from yellow to dark brown, indicating oxidation of the o-aminophenol groups, and c) no striking increase in the amount of arsenoxide; the amount formed is entirely inadequate to account for the change in iodine titer mentioned above. Thus, the oxidation process seems more complicated than can be formulated in terms of simple concepts.

The *in vitro* experiments demonstrate that ascorbic acid does not affect the rate of formation of arsenoxide from neoarsphenamine. It is capable of retarding somewhat the oxidation of o-aminophenol groups, but if the oxidation is continued long enough the end result is the same whether ascorbic acid is present or not. Ascorbic acid also retards the oxidation of the o-aminophenol group in arsenoxide.

The results of the *in vitro* experiments have been confirmed biologically. Thus in a solution of arsenoxide protected by ascorbic acid and exposed to the air there is a continuous decrease in toxicity with time, attributable to the oxidation of As^{3+} to As^{5+} . On the other hand, in a solution of arsenoxide unprotected by ascorbic acid there is an early increase in toxicity due to oxidation of the o-aminophenol group, followed by a decrease in toxicity as the arsenic atom is oxidized. Except at the outset the arsenoxide-ascorbic acid combination is always less toxic and less colored than the arsenoxide alone.

In aged neoarsphenamine solutions the situation is more complex. Initially the neoarsphenamine solution is about 40 per cent more toxic than a similar solution containing ascorbic acid. Both solutions become more toxic on exposure to air, but at about the same rate. If a neoarsphenamine solution is aged for three days in the absence of ascorbic acid, it becomes quite deeply colored and very toxic. However, if ascorbic acid is now added to it, it is no more toxic than a neoarsphenamine solution which contained the same amount of ascorbic acid during the time of the exposure to air. The ascorbic acid, therefore, does not actually prevent to any significant degree the chemical changes which cause neoarsphenamine to become more toxic on exposure to the air, but rather acts by detoxifying *in vivo* the neoarsphenamine which remains unchanged after the oxidation has been stopped. During the exposure to air the ascorbic acid probably remains essentially unchanged.

The *in vitro* experiments thus shed little definite light on the mechanism of ascorbic acid action. They demonstrate that the acid is capable of retarding the oxidation of o-aminophenol groups, but that it does not prevent or retard arsenoxide formation.

The *in vivo* experiments demonstrate that a high blood concentration of ascorbic acid is necessary during the time the neoarsphenamine is in the circulation if the detoxifying effect is to be observed. This may be related to the fact that the presence of an excess of ascorbic acid decreases the redox potential of the blood. However, redox potential is not the only consideration since both lactic

acid and cysteine, which have lower redox potentials than ascorbic acid (22, 23), are inferior to ascorbic acid as detoxicants (1).

The *in vivo* oxidation of neoarsphenamine may affect the o-aminophenol groups primarily, if the analogy to the *in vitro* experiments is preserved. However, in the blood catalysts such as copper, iron, and hemoglobin are present and may alter the behavior of the ascorbic acid. About the only generalization which can be made is that ascorbic acid tends to have its greatest detoxifying effect on those arsenicals which are most susceptible to oxidation *in vitro*.

SUMMARY

A study of the *in vitro* and *in vivo* behavior of solutions of arsenicals, with and without ascorbic acid, has been made. Ascorbic acid appears to be capable of retarding oxidation of the arsenicals, particularly as it involves the labile o-aminophenol groups. It is thought that the administration of ascorbic acid with an arsenical decreases the oxidation-reduction potential of blood sufficiently to prevent immediate oxidation of the arsenical. *In vivo* evidence suggests that the site of the detoxication process is the blood stream, not the tissues such as liver and kidney. It is confirmed that ascorbic acid does not affect the retention of arsenic by liver and kidney.

A method for the analysis of a mixture containing neoarsphenamine, arsenoxide, and ascorbic acid is given. It is based on iodine titrations and physical separation of the neoarsphenamine by means of cetyl pyridinium chloride.

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DIBUTOLINE¹

I. PHARMACODYNAMIC ACTIONS OF A CHOLINE ESTER WITH ATROPINE-LIKE PROPERTIES

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In the quest for an improved drug for use in refraction and internal examination of the eye, Swan and White (1943) were prompted to explore the possibility of synthesizing surface-active derivatives of a choline compound by replacing the hydrophilic $-NH_2$ group of carbaminoylecholine with water-insoluble amines. It was thought that changing thus the molecular structure and physical properties of the substance might alter its physiologic effects. The results of their investigations may be traced in a series of notable papers (1-5). The research culminated in the synthesis of a new class of autonomic drugs: a series of choline esters with mydriatic and cycloplegic action. The addition of the non-polar groups to carbaminoylecholine effected a spectacular reversal of action—"an event unparalleled in autonomic pharmacology."

A number of preparations were synthesized. Of these, dibutyl-urethane of dimethyl-ethyl- β -hydroxyethyl ammonium sulfate (dibutoline)² was found to be the most suitable for clinical ophthalmologic use. The structural relation of this preparation to carbaminoylecholine is shown in figure 1.

The physical properties of dibutoline and its ocular pharmacology have been reported (1-5). This study is concerned with systemic aspects of its pharmacologic actions.

PREPARATIONS AND EXPERIMENTAL METHODS. The stock solution which was used was a 5% solution of dibutoline in distilled water. Decomposition was tested for by checking the hydrogen ion concentration of the solution with litmus paper (3). When a less concentrated solution was desired, a suitable dilution with physiologic saline was prepared. The other drugs which were used in this study were 1. Ether or Veteriaary Nembutal (Abbott Laboratories) for anesthesia. 2. Heparin (Abbott Laboratories) as an anticoagulant. 3. Atropine sulfate and scopolamine hydrobromide. 4. Acetylcholine chloride (Hoffman-La Roche), Prostigmin methylsulfate (Hoffman-La Roche) and Acetyl-beta-methylcholine chloride or Mecholyl (Merck and Co.). 5. Adrenaline chloride (Parke, Davis and Co.).

An indication of the therapeutic range of dibutoline was determined by testing for acute toxicity in anesthetized and unanesthetized dogs. Chronic toxicity studies were not done and the MLD for any of the various laboratory animals has not been determined.

In a series of acute experiments, the systemic aspects of the pharmacologic actions of intravenous injections of dibutoline were studied in dogs anesthetized with nembutal or ether. Healthy unselected stock dogs weighing 8 to 13.7 kilograms served as experimental

¹We obtained the dibutoline which was used in this study through the generosity of Doctor Kenneth C. Swan, Professor of Ophthalmology, University of Oregon Medical School.

²Merck and Company.

animals. Sixteen dogs were used in this study. Femoral blood pressure was recorded with a mercury manometer. In some experiments, heparin was used to minimize the occurrence of intra-cannular clots. The studies were done in intact animals, left vagus dogs and after double vagotomy. Myocardiographic records were obtained in open chest experiments. Vagus blocking effects were determined by faradization of the peripheral end of the cut right vagus nerve, or by electrical stimulation of the intact nerve. Respiratory movements were recorded by a pneumograph which was attached to an air tambour. In several experiments, a submaxillary duct was exposed and cannulated and a drop record of salivary secretion was obtained. The chorda tympani on the side of the cannulated duct was exposed for electrical stimulation, and the lingual nerve was sectioned distal to the chorda tympani branching.

The pupillary reactions to intra-carotid injections of the drug and stimulation of the vago-sympathetic trunk after the administration of dibutoline were studied in a number of instances.

The stability of dibutoline in blood was tested in a few experiments. Prior to this, all intravenous injections were done by a two-syringe technique in order to avoid mixing the drug with venous blood. The criteria of blood pressure response to a 1 cc. test dose and vagus blocking effects were used as indicators of drug activity. At ten minute intervals, a

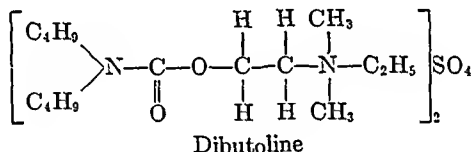
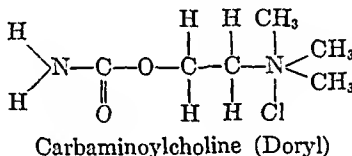


FIG. 1

fractional sample of a dibutoline-blood-oxalate mixture was injected into a femoral vein*. The mixture consisted of dibutoline, 25 mgm., potassium oxalate, 5 mgm., venous blood, 2 cc., and enough physiologic saline solution to make 5 cc.

The effects of intravenous injections of dibutoline on the electrical changes in the heart were studied in the intact unanesthetized dog. Four animals were used. The dogs were placed on their side, connected by lead II to the electrocardiograph and ECG records were obtained following the injection of 0.01 to 10 mgm. per kilogram of dibutoline into a radial vein.

Records of the motility of an intestinal segment in the form of a Thiry or a Thiry-Vella loop of the jejunum with nerve pathways intact were taken by the balloon-mercury-manometer method (6). Six dogs were used. The drug reactions of smooth muscle *in vitro* were also studied. A record of the contractions of the isolated small intestine of a rabbit was obtained after suspending it in an oxygenated bath of Locke's solution at pH 7.8 and a temperature of $37.5 \pm 0.2^\circ\text{C}$.

I. EFFECTS OF INTRAVENOUS INJECTIONS ON THE CIRCULATION. In the acute experiments which were done, the minimal effective depressor dose was 0.02 to 0.05 mgm. per kilogram. There was no evidence of a dosage phenomenon. The

arterial pressure typically began to decrease within 15 to 40 seconds after the injection and reached a minimum in from 1 to 3 minutes. Intravenous injections of less than 0.5 mgm. per kilogram resulted in a transient fall in blood pressure, which returned to the pre-injection level within 1 to 5 minutes after a minimum pressure was reached. A more persistent decrease in blood pressure occurred after the administration of higher dosages. A return to the pre-injection level was not always accomplished if more than 1 mgm. per kilogram of dibutoline was

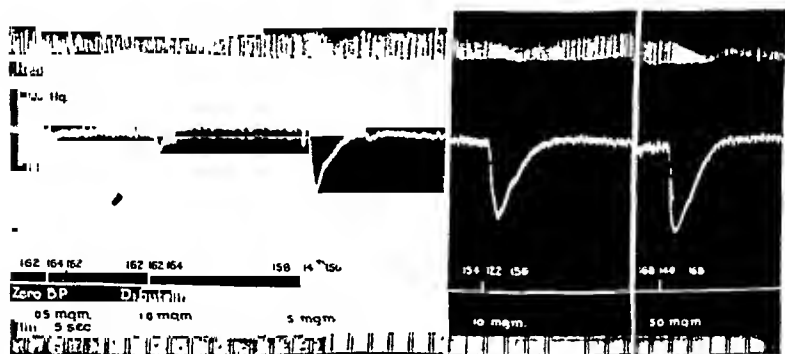


FIG. 2. EFFECT OF DIBUTOLINE ON BLOOD PRESSURE, HEART RATE AND RESPIRATION. Intravenous injections. Intact dog, 10 kilograms. Nembutal intraperitoneal anesthesia. Breaks in record, 3 minutes and 45 minutes. The horizontal row of figures above the zero blood pressure line indicates heart rate, calculated from 30 second intervals. Zero blood pressure line is also procedure line.

TABLE 1

Depressor effect of dibutoline

Average blood pressure decrease after intravenous injection in anesthetized dogs:
16 animals

DOSE	BLOOD PRESSURE DROP
mgm per kg	mm Hg
0.05	3
0.1	12
0.3	24
0.5	27
1.0	38
5.0	67

given. The atropine-like effects of dibutoline in blocking the cardiac vagus and in stopping salivary secretion usually outlasted the decrease in blood pressure which resulted after intravenous injections of the drug. Dibutoline may be injected repeatedly with little or no diminution in the depressor response. The relation of drug dosage to the magnitude of the depressor response is shown in table 1. A typical record is illustrated in figure 2.

A change in heart rate did not occur after the intravenous injection of less than

0.1 mgm. per kilogram of dibutoline. Larger doses resulted in a decrease in the heart rate, which was related directly to the drug dosage. The latency for the onset of cardiac slowing was more than one circulation time. The heart rate was decreased about 10 per cent after 0.5 mgm. per kilogram of dibutoline. A ten-fold increase in drug dosage resulted in a 5 to 20 per cent further decrease in the heart rate. Cardiac irregularities of any kind were notably absent in all of the experiments. The decrease in blood pressure which resulted from 0.5 mgm. per kilogram was not accompanied by a measurable change in the amplitude of the ventricular contractions. Higher doses produced myocardiographic evidence of a decreased cardiac output coincident with the decrease in blood pressure, and both the rate and amplitude of ventricular contractions were lessened.

The depressor response was uninfluenced by vagotomy or atropinization. Similarly, the decrease in heart rate which occurred after intravenous injections of dibutoline in anesthetized animals was not significantly altered by atropinization or double vagotomy.

We have not obtained plethysmographic records, but our results suggest that the blood pressure changes which occur after the intravenous injection of small doses of dibutoline (less than 0.1 mgm. per kilogram) are peripheral in origin. Arterial pressure decreased although the rate and the amplitude of ventricular contractions were unchanged. Moreover, there was no evidence of medullary depression in these experiments and there is a local spasmolytic action of dibutoline on smooth muscle other than the arteriole (cf. intestinal tract).

There was impairment of the inhibitory effect of vagus stimulation after intravenous injection of the minimal depressor dose. With increased dosage, the vagus block became complete and the heart rate response to vagal stimulation was cardiac acceleration with a slight elevation of the blood pressure. The duration of the vagus block was related to dosage. The results of a representative experiment are illustrated in figure 3. This information is summarized in table 2.

The profound cardiac and depressor actions of acetylcholine and acetyl-beta-methylcholine were prevented or abolished by dibutoline. The dibutoline block was not absolute, for it could be overcome by the intravenous injection of more acetylcholine or more acetyl-beta-methylcholine. It was then possible to counteract again the effects of the cholinomimetic drugs by the injection of more dibutoline. The nicotinic effect of a 5 mgm. dose of acetylcholine was unmasked by dibutolinization.

The pressor response to the intravenous injection of 1 cc. of a 2×10^{-5} concentration of adrenaline was unaffected by 1 mgm. per kilogram of dibutoline.

The experiments just described afford evidence for the following conclusions:

1. There is a peripheral site of action of the drug which is comparable to the selective action of atropine on autonomic structures, blocking the muscarinic effects of the cholinomimetic compounds on the heart and the circulation.
2. Dibutoline is not a nicotine-paralyzing drug which blocks pre-ganglionic cholinergic synapses for the nicotinic effect of a large dose of acetylcholine is unmasked not abolished by dibutolinization.
3. It is not adrenolytic or sympathicolytic, since

the pressor response to injected adrenaline is unaltered by it and the reactivity of the iris dilator fibers to electrical stimulation of the cervical sympathetic trunk or to stimulatory drugs is apparently uninfluenced by either the local application of dibutoline (5) or intravenous administration of the drug.

TABLE 2

Vagus blocking action of dibutoline

Dog #11: February 22, 1945. 10 kilogram, left vagus animal. Nembutal anesthesia, 6½ cc. intraperitoneal. Secondary coil of inductorium, 8-00", 2 batteries in primary circuit.

DIBUTOLINE	VAGUS BLOCK	COMPLETE RECOVERY TIME
mgm. per kg.		minutes
0.05	Incomplete	6
0.5	Complete	12
2.5	Complete	62

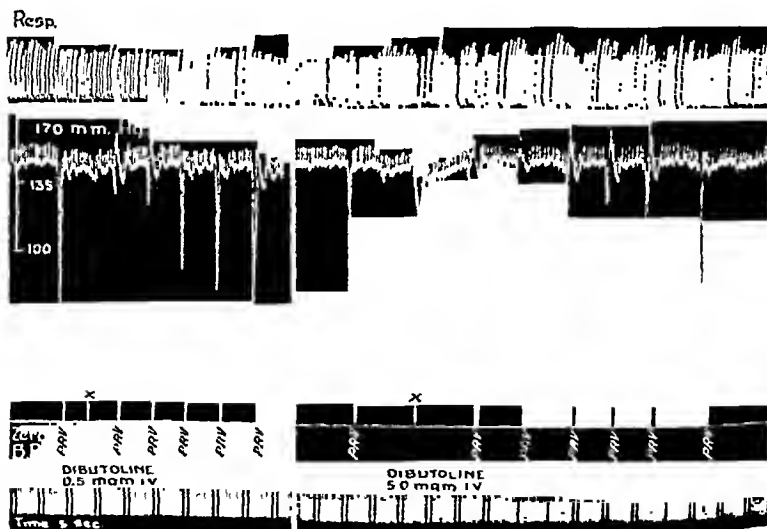


FIG. 3. VAGUS BLOCKING ACTION OF DIBUTOLINE

Left vagus dog, 10 kg. Nembutal intraperitoneal anesthesia. PRV indicates electrical stimulation of peripheral right vagus nerve, secondary coil at 8-00" throughout experiment. Break in record, 20 minutes.

In one type of experiment which was done, the secondary coil of an inductorium was set so that the response to electrical stimulation of the right vagus nerve was cardiac arrest. An identical stimulus was ineffective after the intravenous injection of 0.3 mgm. per kilogram of dibutoline. After one minute a 0.5

mgm. dose of prostigmin was injected intravenously. The vagus nerve was again stimulated after a lapse of one minute and cardiac arrest occurred. The bradycardia and cardiac irregularity which persisted after the nerve stimulation were terminated abruptly by repeating the initial dose of dibutoline.

The most likely explanation for these results is that the dibutoline block does not interfere with the release of acetylcholine at postganglionic cholinergic nerve endings to the heart. Although vagus block occurred after the initial injection of dibutoline, the subsequent administration of prostigmin would allow, then, for the accumulation of sufficient acetylcholine to overcome the dibutoline block when the nerve was again stimulated. A less likely explanation would be that prostigmin exerts some direct effect exclusive of its anti-cholinesterase action.

II. ELECTRICAL CHANGES IN THE HEART. ECG records were obtained following the intravenous injection of dibutoline in intact unanesthetized dogs. The characteristic features of the response to the intravenous administration of 0.01 to 10 mgm. per kilogram were studied.

An initial period of bradycardia after the injection did not occur. The minimal cardio-accelerator dose was more than 0.01 and less than 0.05 mgm. per kilogram. The normal sinus arrhythmia, which is usually exaggerated in dogs, was promptly lost with the onset of the sinus tachycardia. The period of latency for the accelerator response was equivalent to an arm to heart circulation time, i.e., 7 to 9 seconds. The increase in the heart rate developed progressively and was usually maximal within 30 to 90 seconds after the injection. Since the increase in heart rate after high dosages (5 to 10 mgm. per kilogram) exceeded the predicted rise from vagus release alone and dibutoline has been shown to possess a depressor action in the anesthetized animal, it is likely that a part of the cardiac acceleration which occurred after high dosages was on the basis of adrenomimetic substances liberated at adrenergic nerve endings activated reflexly by a fall in blood pressure.

Cardiac irregularities of any kind were not observed in the four animals studied. The significant ECG changes which were noted involved the P wave, the T wave and the S-T segment. There was usually a 0.1 to 0.3 mv. depression of the P wave (or inversion of the P wave after high dosages) which developed within an arm to heart circulation time after the beginning of the injection and persisted for only seconds. The changes in the P wave were reversible in every instance, within 5 minutes or less. The electrocardiographic changes in the T wave and S-T segment were more persistent and still evident 4 to 5 minutes after the injection when a last ECG record was obtained. However, significant alterations in the day to day control records of the different animals were not observed. The various changes in the T wave and S-T segment which occurred were the following: a 0.1 to 0.3 mv. accentuation of a normally inverted T wave, a flattening or reversal of a normally inverted T wave, a 0.1 to 0.5 mv. heightening of an upright T wave, inversion of a normally upright T wave, a diphasic change in the T wave or disappearance of a normally diphasic T wave, or a 0.1 to 0.5 mv. elevation of the S-T segment.

Selected ECG tracings are shown in figure 4. The effect of various intravenous doses of dibutoline in one of the animals is shown in table 3.

III. EFFECTS ON RESPIRATION. Neither the amplitude nor the rate of respiratory excursions was altered by the intravenous injection of less than 0.5 mgm. per kilogram of the drug in the anesthetized animals. Larger doses resulted in a decrease in blood pressure which was associated with a tachypnea. The amplitude of the respiratory excursions was decreased in some of the experiments when the hypotension was severe. The respiratory effects were synchronous with a decrease in arterial pressure and did not otherwise occur. This result is illustrated in figures 2 and 5. The likely explanation for these changes is in terms of reflex activation of the respiratory center by arterial hypotension utilizing the carotid sinus and aortic arch mechanisms. The bradypnea which sometimes occurred

TABLE 3
8.6 kilogram, intact unanesthetized dog

6.6 kilogram, intact unanesthetized dog

DIBUTOLINE I.V.	BASAL HEART RATE	ONSET OF ACCELERATION	INCREASE IN HEART RATE	HEART RATE PER MINUTE AFTER THE DIBUTOLINE INJECTION CALCULATED FROM 10 SEC. INTERVALS				ECG CHANGES
				Seconds after injection				
				10	30	90	300	
mgm per kg.		sec.	%					
0.01	96		4	100	92	92	92	None
0.10	120	7	40	120	150	168	132	
1.0	90	9	173	96	198	246	246	T wave lost normal bi- phasic appearance 10 seconds after injection, recovery in 90 seconds 0.2 to 0.4 mv. inversion of P wave, T wave elevated 0.5 mv.
5.0	96	7	144	126	204	174	234	

during the period of compensatory readjustment of the blood pressure is explainable on the basis of acapnia developed during a hyperventilatory period.

The intra-carotid injection of 0.05 to 0.5 mgm. per kilogram of dibutoline did not produce respiratory effects which were qualitatively different than those already described.

There were no significant respiratory effects from the intravenous injection of 0.01 to 10 mgm. per kilogram of dibutoline in unanesthetized animals.

IV. EFFECT ON SALIVARY SECRETION. The subcutaneous or intravenous administration of dibutoline produced a dry mouth in the experimental animals which were studied. The influence of dibutoline on the action of the chorda tympani and the secretory response to the injection of a cholinomimetic drug was studied.

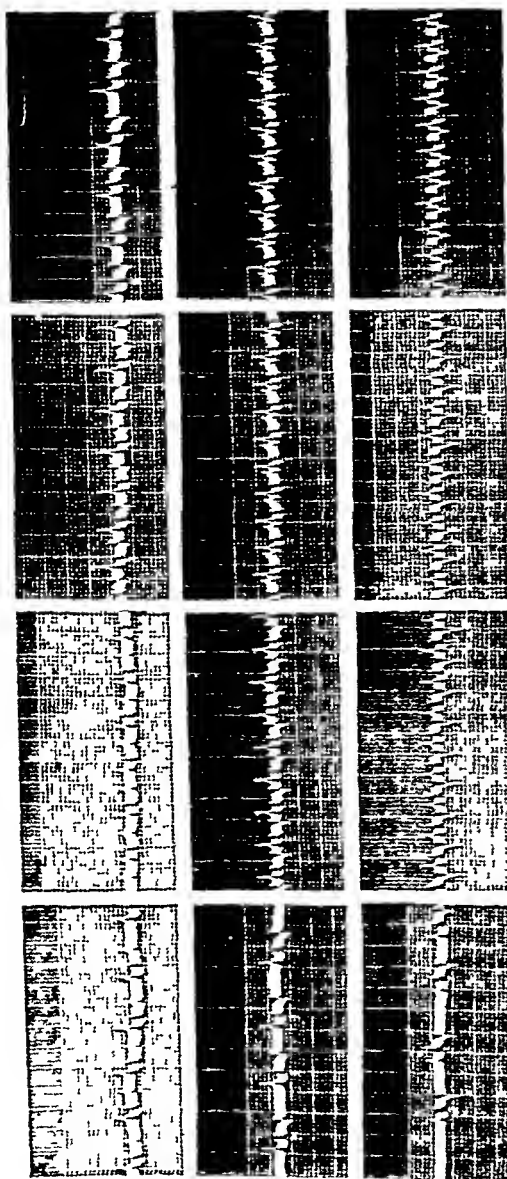


Fig. 1. ECG TRACINGS FROM DIFFERENT ANIMALS, INTACT UNANESTHETIZED DOGS

A_1 control record, 0.1 mgm per kg of dibutoline between A_1 and A_2 . Records A_2 : 1 at 30, 90 and 300 seconds after the injection of dibutoline, respectively. Note P wave depression in A_2 , depression of S-T segment and biphasic T wave in A_2 , which persists through record A_4 . B_1 control record, 1 mgm per kg of dibutoline between B_1 and B_2 . Records B_2 : 1 as above. Note elevation of S-T segment and disappearance of normally biphasic T wave, with appearance of upright T wave, in B_2 . C_1 control record, 5 mgm per kg of dibutoline between C_1 and C_2 . Records C_2 : 1 as above. Note accentuation of normally negative T wave in C_2 . Heart rate calculated from 10 second intervals in upper right corner of record

The increase in salivary secretion which resulted from strong stimulation of the chorda tympani was abolished by a 0.3 mgm. per kilogram dose of dibutoline. This dose of the drug also produced a complete block of the cardiac vagus, a 10 to 15 per cent decrease in the blood pressure and a decrease in heart rate. It is notable that recovery from the circulatory effects of the injection and a return of the normal heart rate response to peripheral vagal stimulation occurred before the inhibitory effects of the injection on salivary secretion had disappeared. The protocol of a typical experiment is presented in table 4.

The profuse salivatory response to acetyl-beta-methylcholine and the depressor action of a 0.015 mgm. per kilogram dose of the drug were nullified immediately by 0.2 mgm. per kilogram of dibutoline. In other experiments, the cardiac, cir-

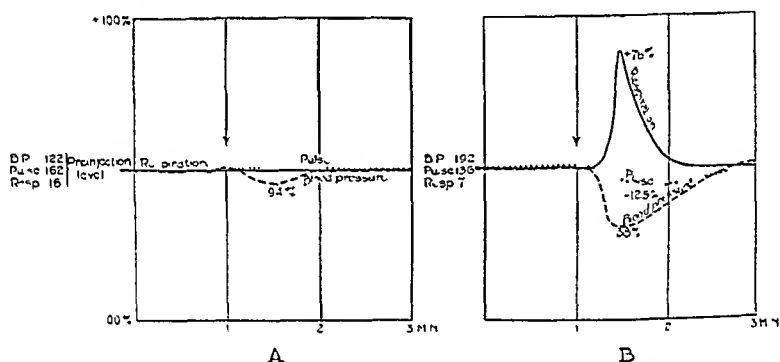


FIG 5 EFFECT OF DIBUTOLINE ON BLOOD PRESSURE, HEART RATE AND RESPIRATION

A Intact dog, 10 kg Nembutal intraperitoneal anesthesia Dibutoline, 0.1 mgm per kilogram by intravenous injection, at arrow. Note decrease in blood pressure without change in heart rate or respiratory rate

B Intact dog, 9.1 kg Nembutal intraperitoneal anesthesia Dibutoline, 1 mgm per kilogram by intravenous injection, at arrow. Note the synchronous effects on blood pressure and respiratory rate, and the comparatively slight decrease in heart rate.

culatory and secretory responses to the injection of acetyl-beta-methylcholine were prevented by dibutoline

V. EFFECT ON THE ISOLATED RABBIT INTESTINE. It was shown by the isolated intestine technique that there is a direct inhibitory action of dibutoline on the smooth muscle of the intestinal wall as well as an atropine-like action, blocking the effects of acetylcholine

The smallest effective concentration in these experiments was less than 2×10^{-6} . In this dosage, equivalent concentrations of dibutoline and atropine produced similar inhibitory effects on the tone and rhythmic contractions of the isolated segment of intestine.

The increased tone and rhythmic motility which resulted from the addition of a 1×10^{-7} concentration of acetylcholine to the bath solution was abolished by dibutoline in a 2×10^{-6} concentration. The type of record obtained is illustrated in figure 6. A high concentration was required to influence the spasm induced by

a 2×10^{-4} concentration of barium chloride. A 4×10^{-5} concentration possessed a distinct though incomplete antispasmodic action. An equivalent dose of atropine was ineffective against the intestinal effects of barium chloride.

VI. EFFECT ON THE DOG INTESTINE IN SITU. The results of these studies are qualitatively consistent with the results of the *in vitro* experiments which have been described. All injections were made by the intravenous route.

After a latent period of 12 to 30 seconds, the injection of 0.01 to 1 mgm. per kilogram of dibutoline resulted in a reduction in the amplitude of rhythmic contractions of the intestinal segment and a sharp decrease in tonus. The maximal

TABLE 4

Effect of dibutoline on the action of the chorda tympani

Dog #2: October 24, 1944. 10 kilogram, left vagus animal. Ether anesthesia. Dibutoline, 0.3 mgm. per kilogram intravenously.

TIME	STIMULATIONS/INJECTIONS	SALIVA	BLOOD PRESSURE
<i>minutes</i>		<i>glt</i>	<i>mm. Hg</i>
0	Stimulate chorda tympani, secondary coil at 8-00", two batteries in primary circuit		84
0-4		22	
5	3 mgm. of dibutoline, i.v.		
6		2	70
7½	Vagus block complete		
8½	Stimulate chorda tympani	0	74
9½	Vagus block incomplete		
10½	Stimulate chorda tympani	0	82
12	Vagus block absent		
12½	Stimulate chorda tympani	1	84
13½	Stimulate chorda tympani	2	
15	Stimulate chorda tympani		
15½		2	84
18	Stimulate chorda tympani	4	
19½		2	
20½	Stimulate chorda tympani		
21		2	
22		4	
37	Stimulate chorda tympani	13	94

effects of the injection were observed immediately. The intestinal segment did not show a hyper-motile recovery phase. There was a progressive return of tonus and motility to the pre-injection state. The amplitude of rhythmic contractions usually showed a gradual increase to normal which preceded the recovery of tonus.

The minimal effective dose for exerting an inhibitory action on tonus and motility was near 0.01 mgm. per kilogram.

The duration as well as the degree of intestinal inhibition varied with the dose of dibutoline used. The effects of 0.01 mgm. per kilogram of the drug were observable for less than 5 minutes. With this dosage, inhibitory effects were

usually submaximal and amotility did not always occur. A 0.1 mgm. per kilogram dose produced amotility of the intestinal segment for about 1 minute with a proportionately greater decrease in tonus. The amplitude and rate of rhythmic contractions were decreased for about 15 minutes. A 1 mgm. per kilogram dose of the drug resulted in even more prolonged inhibitory effects, which lasted for 20 to 45 minutes.

A comparison of responses to equivalent doses of dibutoline and atropine was made. The type of record obtained is illustrated in figure 7. Dibutoline produced a more marked degree of intestinal inhibition during the time of its maxi-

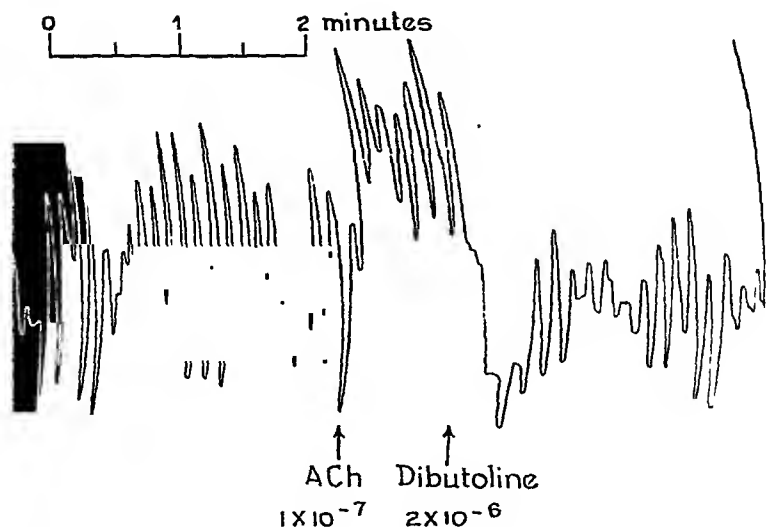


FIG. 6. ISOLATED RABBIT INTESTINE

mal effect than did atropine in most of the experiments, but there was more rapid recovery from the dibutoline inhibition.

The increased tonus or spasm which result from the administration of a test dose of prostigmin may be counteracted and subnormal tonus and motility produced by the injection of dibutoline. A typical response is shown in figure 8. The dose of dibutoline needed was small and similar to that which was required to inhibit the intestinal segment when a test dose of prostigmin had not been given. The degree of subnormal tonus and motility which resulted was as great as after the injection of an equivalent dose of atropine. However, the antagonism of the prostigmin effect by dibutoline was of shorter duration than that from an equivalent dose of atropine. The response to a large test dose of prostigmin was



FIG. 7. COMPARISON OF THE EFFECT OF DIBUTOLINE AND ATROPINE IN EQUIVALENT DOSES ON THE MOTILITY OF AN INTESTINAL SEGMENT
Intact unanesthetized dog, 8.6 kg

prevented for a time by the injection of a comparatively small dose of dibutoline (less than 0.05 mgm. per kilogram).

Although it completely opposed the effects of prostigmin on intestinal motility, the excitatory action on tonus and rhythmic contractions of a test dose of morphine was relatively little affected by dibutoline. The type of record obtained is illustrated in figure 9. This inability to counteract the intestinal effects of morphine was evident although high dosages of dibutoline were given. The antispasmodic effect of a 0.05 mgm. per kilogram dose of the drug exceeded that of an equivalent dose of atropine.

The amount of dibutoline by intravenous injection that is required to inhibit intestinal motility in the unanesthetized dog is less than the minimal needed for the production of cardiac acceleration. This fact was observed repeatedly by periodic determinations of the heart rate while recording the response of an in-

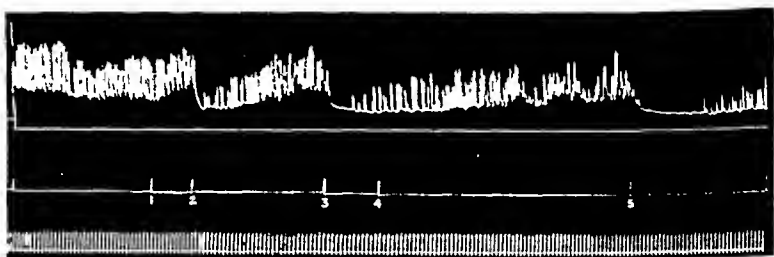


FIG. 8. DIBUTOLINE ANTAGONISM OF PROSTIGMIN SPASM OF AN INTESTINAL SEGMENT

From above downward there is shown 1, balloon-mercury-manometer record from the distal end of a jejunal Thiry-Vella loop in an unmedicated, unanesthetized dog; 2, zero pressure level; 3, record of procedures; 4, time in 10 second intervals. Marks indicate intravenous injections as follows: (1) 0.12 mgm. of prostigmin, (2) 0.5 mgm. of dibutoline, (3) 5 mgm. of dibutoline, (4) 0.12 mgm. of prostigmin and (5) 0.5 mgm. of atropine sulfate. 8.6 kg. animal.

testinal segment to injections of the drug, and by obtaining simultaneous records of electrical changes in the heart and of intestinal motility.

We have not studied directly the effect of dibutoline on bronchiolar muscle, but it was noted in these experiments that the broncho-constrictor action of a test dose of prostigmin was blocked by dibutoline.

In intact unanesthetized loop dogs, the fascicular skeletal muscle tremors which appeared after the injection of a large test dose of prostigmin were unaffected by 0.5 mgm. per kilogram of dibutoline.

VII. EFFECT OF INTRA-CAROTID INJECTION. A number of intra-carotid injections were done in the acute experiments which have been described. 0.05 to 0.5 mgm. per kilogram of dibutoline was administered. The injection of 0.5 mgm. per kilogram produced a typical depressor response which was equal to or less than the effect of an equivalent dose by intravenous injection. No respiratory effects were observed in these experiments. A submaximal, ipsilateral dilatation of the pupil occurred. It was possible to produce a maximal dilatation by faradization of the vago-sympathetic trunk.

VIII. STABILITY IN BLOOD. A few experiments were done in an attempt to test for inactivation of dibutoline by blood. The criteria of blood pressure decrease and vagus blocking action of a test dose were used as indications of persistent drug activity. The studies gave negative results. There was no apparent in-

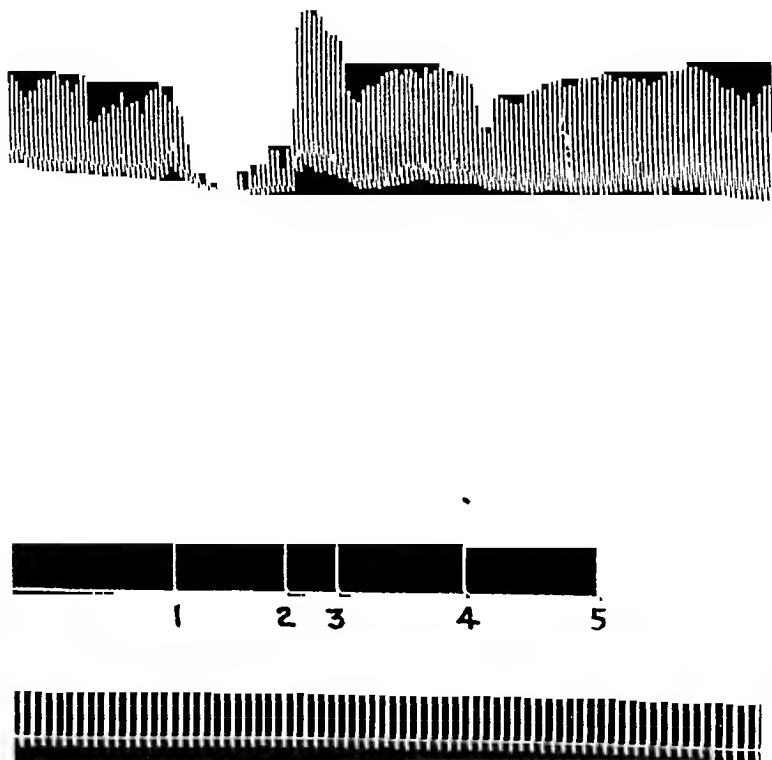


FIG 9. INTESTINAL EFFECTS DIBUTOLINE VS MORPHINE

Writing points arranged as in figure 8, except that motility record is from a jejunal Thiry loop. Marks indicate intravenous injections as follows (1) 0.1 mgm of dibutoline, (2) 4 mgm of morphine sulfate, (3) 1 mgm of dibutoline, (4) 10 mgm of dibutoline and (5) 1 mgm of atropine sulfate. 9.2 kg dog.

activation of the drug by blood within one hour after the preparation of a blood-oxalate-dibutoline mixture.

IX. EFFECT OF HYPODERMIC AND INTRAVENOUS INJECTION IN UNANESTHETIZED DOGS. Swan and White (5) have reported the minimal lethal dose of dibutoline administered intraperitoneally in rabbits and rats as approximately 75 mgm. per kilogram.

In the acute experiments, it was evident that the difference between the mini-

mal effective dose and the minimal toxic dose must be very great. Repeated high dosages of the drug resulted in little or no adverse effect, other than the blood pressure and heart rate changes which occurred. As much as 300 mgm. of dibutoline was given in the course of a two-hour experiment without producing a lethal result. This was the largest total dosage given.

The low toxicity of dibutoline was also apparent after the subcutaneous and intravenous injection of 0.01 to 10 mgm. per kilogram of the drug in intact unanesthetized animals. The intravenous administration of dibutoline was as well tolerated as the subcutaneous injection of equivalent doses. High dosages (1 to 10 mgm. per kilogram) were as well borne as the injection of the minimal effective dose for producing intestinal inhibitory effects (less than 0.01 mgm. per kilogram).

TABLE 5

Effects of subcutaneous injections of dibutoline in unanesthetized dogs

EXPERIMENT NUMBER	MGm. PER KG.	HR (HEART RATE) AND P (DIAMETER OF PUPIL)	HEART RATE CALCULATED FROM 30 SECOND INTERVALS										REMARKS	
			Pre-injection	Minutes after injection										
				1	2	3	5	10	15	30	45	60		
I, 3	0.05	mm. HR	144	136	148	170	178	196	176	136				
II, 1	0.5	HR P	130 (2)	128	118 (2)	122	142 (2)	214	180	166 (2)				
III, 2	1.0	HR	112	112	108	114	128	198	214	186	164			
II, 2	2.5	HR P	106 (2)	90 (2)		100 (2)	170 (3½)	240	236 (3½)	232	232 (10)		Hyperactive reflexes	
IV, 1	5.0	HR P	92 (3)	88	88 (3)	126	216 (3)	262	240 (3)	228 (8)	204 (5)	174 (4)	Hyperactive reflexes	

We have not determined the minimal toxic dose for dibutoline, but exceeding the effective dose for producing intestinal effects by more than 1000 times did not result in symptoms of overdosage. The animals typically developed a dry mouth, pupillary dilatation and loss of accommodation. There was a marked sinus tachycardia and a disappearance of the normal sinus arrhythmia. The only changes which could be attributed to high doses of dibutoline (and might be indicative of overdosage) were restlessness, hyperactivity of the deep tendon reflexes and reversible and minimal ECG changes.

After subcutaneous injections of dibutoline in unanesthetized dogs there seemed to be a mild cardio-inhibitor action of the drug before the onset of cardiac acceleration. This finding was constant in the limited number of observations which were made. Since the degree of deceleration which occurred was slight and the series of determinations was small, the question of a biphasic action on the heart rate and a dosage phenomenon has not been answered satisfactorily by

these experiments. Data from various dosages in different animals shown in table 5 illustrate the results obtained.

A depressor response did not occur in anesthetized dogs after subcutaneous injections of dibutoline, even in high dosage. 2.5 mgm. per kilogram produced no change in blood pressure although it resulted in complete vagus block.

SUMMARY AND CONCLUSIONS

The combined results of this and of previous other studies which have been reported provide a basis for a description of the pharmacodynamic actions of dibutoline.

Dibutoline possesses pharmacologic actions which are qualitatively identical with the autonomic-blocking action of atropine. In adequate dosage it prevents or abolishes the muscarinic effects of injected choline derivatives. It blocks the effects of endogenously produced acetylcholine at effector cells innervated by postganglionic cholinergic nerves, i.e., 1. The iris constrictor. 2. The cardiac vagus. 3. The submaxillary gland. 4. Arteriolar smooth muscle. 5. Smooth muscle of the intestinal tract.

In equivalent doses, the duration of its action is shorter than for atropine; however, prolonged effects without toxicity were obtained by increasing the drug dosage.

Autonomic-blocking effects of dibutoline were evident after the administration of doses which did not produce a decrease in blood pressure or a change in the heart rate.

In this series of experiments, the following illustrative results were obtained:

a) Less than 0.01 mgm. per kilogram by intravenous injection exerted an inhibitory action on tonus and rhythmic contractions of an intestinal segment in the intact unanesthetized dog. This was less than the minimal cardio-accelerator dose for the same animals.

b) A 2×10^{-6} concentration of dibutoline was found to lessen the tone and decrease the amplitude of rhythmic contractions of an isolated segment of rabbit intestine in a drug-free bath.

c) A 0.02 mgm. per kilogram dose by intravenous injection produced vagus blocking effects in the anesthetized dog; less than 0.05 mgm. per kilogram was effective in the unanesthetized animal.

d) In the acute experiments, 0.3 mgm. per kilogram of dibutoline blocked completely the effect of strong stimulation of the chorda tympani. The secretory and circulatory effects of a 0.25 mgm. dose of acetyl-beta-methylcholine were abolished by 0.2 mgm. per kilogram of dibutoline.

The results of the prostigmin experiments would indicate that the release of acetylcholine at postganglionic vagus endings to the heart is not prevented by dibutolinization.

The stability of dibutoline in blood was tested. There was no evidence of inactivation of the drug within an hour's time.

That dibutoline is not adrenolytic or sympathicolytic and depressant to effector cells innervated by postganglionic adrenergic nerves, or nicotine-paralyzing and

blocking preganglionic cholinergic synapses, is suggested by the following facts. The pressor response to the intravenous injection of adrenaline was unaltered by dibutoline. Moreover, the iris dilator fibers appear to be unaffected by the systemic administration of dibutoline as well as by its local application. The nicotinic action of acetylcholine in high dosage is unmasked by dibutolinization.

We have not obtained plethysmographic records, but it seems likely that the blood pressure decrease after the intravenous injection of dibutoline is due in part to a decrease in arterial resistance. When small doses were given (less than 0.1 mgm. per kilogram) a blood pressure decrease occurred without producing a change in the rate or the amplitude of ventricular contractions. The minimal depressor dose was 0.02 to 0.05 mgm. per kilogram. The response was not abolished by double vagotomy or by atropinization. Dibutoline in high dosage by subcutaneous injection resulted in no significant alteration in blood pressure, but exerted its autonomic-blocking action on smooth muscle and secretory glands.

The typical cardiac response to the intravenous injection of dibutoline in anesthetized dogs was deceleration. This result was observed repeatedly. The minimal effective dose for producing a decrease in the heart rate was near 0.1 mgm. per kilogram. A decrease of as much as one-third resulted from the intravenous injection of 5 mgm. per kilogram. Cardiac slowing was not abolished by vagotomy or atropinization. The amplitude as well as the rate of ventricular contractions decreased after a 0.5 mgm. per kilogram dose of dibutoline.

In the intact unanesthetized animal, a period of prolonged cardioacceleration resulted from the subcutaneous or intravenous injection of dibutoline. In some instances, the increase was more than 170 per cent. A biphasic effect on the heart rate was evident after subcutaneous injections of the drug, but a limited and therefore unconvincing number of experiments were done. There was a short initial phase of bradycardia, which was followed by the onset of a persistent and marked sinus tachycardia.

Cardiac irregularities were conspicuously absent in all of the experiments. Significant ECG changes did not develop after the injection of less than 0.1 mgm. per kilogram of dibutoline. Changes ascribable to the effect of dibutoline were minimal and reversible after the injection of higher dosages in intact unanesthetized dogs.

Studies of the isolated rabbit's intestine indicate that dibutoline possesses a direct spasmolytic effect on intestinal muscle as well as an atropine-like action.

A remarkable feature of dibutoline is its extremely low toxicity. It was possible to exceed by more than 1000 times the minimal effective dose for producing an inhibitory action on the tonus and rhythmic motility of the intestine without reaching the minimal toxic dose. The only indications of possible overdosage in intact unanesthetized dogs after the subcutaneous or intravenous administration of 0.01 to 10 mgm. per kilogram of dibutoline were restlessness, hyperactive deep tendon reflexes and minimal and reversible ECG changes. In no instance was a lethal result effected.

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blocking preganglionic cholinergic synapses, is suggested by the following facts. The pressor response to the intravenous injection of adrenaline was unaltered by dibutoline. Moreover, the iris dilator fibers appear to be unaffected by the systemic administration of dibutoline as well as by its local application. The nicotinic action of acetylcholine in high dosage is unmasked by dibutolinization.

We have not obtained plethysmographic records, but it seems likely that the blood pressure decrease after the intravenous injection of dibutoline is due in part to a decrease in arterial resistance. When small doses were given (less than 0.1 mgm. per kilogram) a blood pressure decrease occurred without producing a change in the rate or the amplitude of ventricular contractions. The minimal depressor dose was 0.02 to 0.05 mgm. per kilogram. The response was not abolished by double vagotomy or by atropinization. Dibutoline in high dosage by subcutaneous injection resulted in no significant alteration in blood pressure, but exerted its autonomic-blocking action on smooth muscle and secretory glands.

The typical cardiac response to the intravenous injection of dibutoline in anesthetized dogs was deceleration. This result was observed repeatedly. The minimal effective dose for producing a decrease in the heart rate was near 0.1 mgm. per kilogram. A decrease of as much as one-third resulted from the intravenous injection of 5 mgm. per kilogram. Cardiac slowing was not abolished by vagotomy or atropinization. The amplitude as well as the rate of ventricular contractions decreased after a 0.5 mgm. per kilogram dose of dibutoline.

In the intact unanesthetized animal, a period of prolonged cardio-acceleration resulted from the subcutaneous or intravenous injection of dibutoline. In some instances, the increase was more than 170 per cent. A biphasic effect on the heart rate was evident after subcutaneous injections of the drug, but a limited and therefore unconvincing number of experiments were done. There was a short initial phase of bradycardia, which was followed by the onset of a persistent and marked sinus tachycardia.

Cardiac irregularities were conspicuously absent in all of the experiments. Significant ECG changes did not develop after the injection of less than 0.1 mgm. per kilogram of dibutoline. Changes ascribable to the effect of dibutoline were minimal and reversible after the injection of higher dosages in intact unanesthetized dogs.

Studies of the isolated rabbit's intestine indicate that dibutoline possesses a direct spasmolytic effect on intestinal muscle as well as an atropine-like action.

A remarkable feature of dibutoline is its extremely low toxicity. It was possible to exceed by more than 1000 times the minimal effective dose for producing an inhibitory action on the tonus and rhythmic motility of the intestine without reaching the minimal toxic dose. The only indications of possible overdosage in intact unanesthetized dogs after the subcutaneous or intravenous administration of 0.01 to 10 mgm. per kilogram of dibutoline were restlessness, hyperactive deep tendon reflexes and minimal and reversible ECG changes. In no instance was a lethal result effected.

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THE RATE OF ACTION OF SULFADIAZINE AND QUININE ON THE MALARIAL PARASITE, *PLASMODIUM GALLINACEUM*

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Numerous investigators have found that sulfanilamides do not produce complete inhibition of bacteria immediately but that a more or less extensive time lag occurs (1). Although Libby (2) found appreciable growth during the first hours of treatment his sensitive technique of photorefractometric turbidity readings showed that population increase of several species of bacteria was retarded fairly early. He suggested that progressive reduction of the growth rate occurs before complete inhibition of development is attained.

It seems possible that the length of time required for effective inhibition by sulfanilamides or other slowly acting compounds varies inversely with the growth and multiplication rate of the organism. This has obvious implications for the length of treatment necessary in chemotherapeutic trials with more complex and slowly multiplying organisms such as the protozoa. We have used the malarial organism, *Plasmodium gallinaceum*, in chickens to test this hypothesis. Sulfadiazine was chosen since it is more or less active against various species of avian and human malaria (3, 4, 5). Quinine was chosen as a standard for comparison.

METHODS. The parasites were introduced intravenously into fifteen uninfected one-week-old chickens (single comb white Leghorn cockerels) two days after drug treatment began. Thus, untreated parasites were suddenly placed in an environment containing the drugs. Although the parasites from blood-induced infections of this species are not very synchronous in development, a homogeneous inoculum of small forms was obtained by using the blood from a bird seven days after intravenous injection with sporozoites. The treated birds were kept on drug diets and continuous light throughout the experiment to insure the maintenance of relatively constant blood levels (3). Five birds were treated with sulfadiazine, five with quinine and five were not treated. Each drug constituted 0.2 per cent of the diet, a concentration at least four times that which shows maximum activity in our routine experiments (cf. discussion). The average free sulfadiazine concentration in the blood on the third day (one day after infection) was 7.0 mgm./100 ml., with a range of 5.4 to 8.5 mgm.%. A level of 1 mgm./100 ml. gives maximum activity in our routine drug tests.

Parasite counts on the test birds were made immediately after they were inoculated. About one half of one per cent of the red blood cells contained parasites at that time. Additional blood smears were made from each bird at twelve hour intervals thereafter. Except in a few instances where the parasites were too low in number, the counts were based on observations of fifty or more parasites, a number sufficiently large to insure a standard error of less than 10 per cent of the parasite count. Once each day a red blood cell count was made on each bird and the parasite counts put on a volume basis, i.e., number of parasites per cubic millimeter of blood. In order to detect any influence the drugs might have on growth or reproduction, differential counts of the parasites were made. The parasites were classified by their size (in relation to the size of the nucleus of the infected erythrocyte) and also by the number of parasite nuclei present, as follows: class A) "small forms", i.e.,

less than one-half the size of the erythrocyte nucleus; class B) smaller than the erythrocyte nucleus but larger than one-half its size; class C) larger than the nucleus but with less than eight parasite nuclei; class D) parasites with more than eight nuclei.

RESULTS. The curves of the parasite populations throughout the test period are plotted in fig. 1. In fig. 2 are plotted the curves of the percentage of small parasites (class A) and the percentage of large parasites (classes C and D combined) with time. The intermediate class B is omitted for purposes of simplification. The life cycle of the erythrocytic stage of *Plasmodium gallinaceum* is about thirty-six hours in length, as evidenced by the interval between the peaks of the curves of both the small and the large forms and the population growth

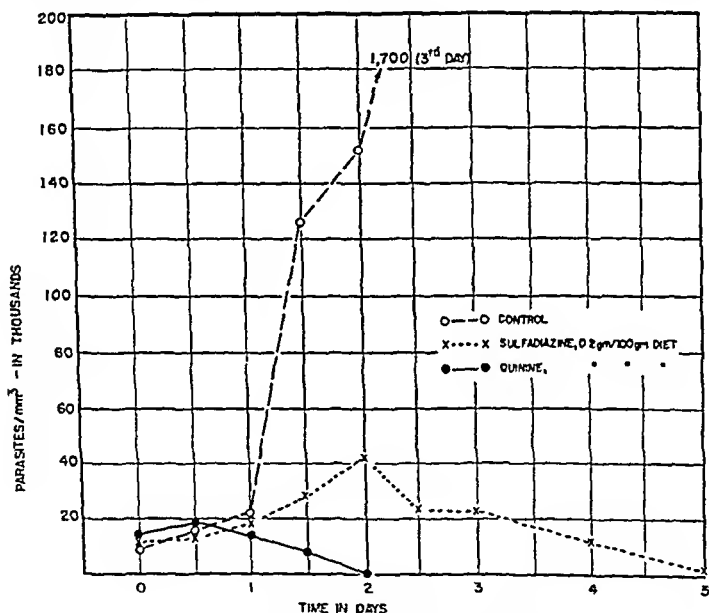


FIG. 1. THE EFFECT OF SULFADIAZINE AND QUININE ON POPULATIONS OF *P. GALLINACEUM*. EACH VALUE REPRESENTS FIVE BIRDS

of the controls. This confirms the earlier reports summarized by Beltran (6) and the recent report of Taliaferro, Coulston and Silverman (7).

High levels of sulfadiazine did not completely suppress the growth of the parasites for at least two and one-half to three days (fig. 2). During this time there were two sharp drops in the percentage of small rings accompanied by two marked increases in the percentage of large parasites. In contrast, the parasites treated with quinine remained small in four birds and did not develop beyond the four-nucleate stage in the fifth bird. Sulfadiazine did not prevent merozoite formation or their invasion of erythrocytes as shown by the sudden increase in parasitemia and in the percentage of small rings between one and one

and one-half days, concurrently in the sulfadiazine treated birds and the untreated controls.

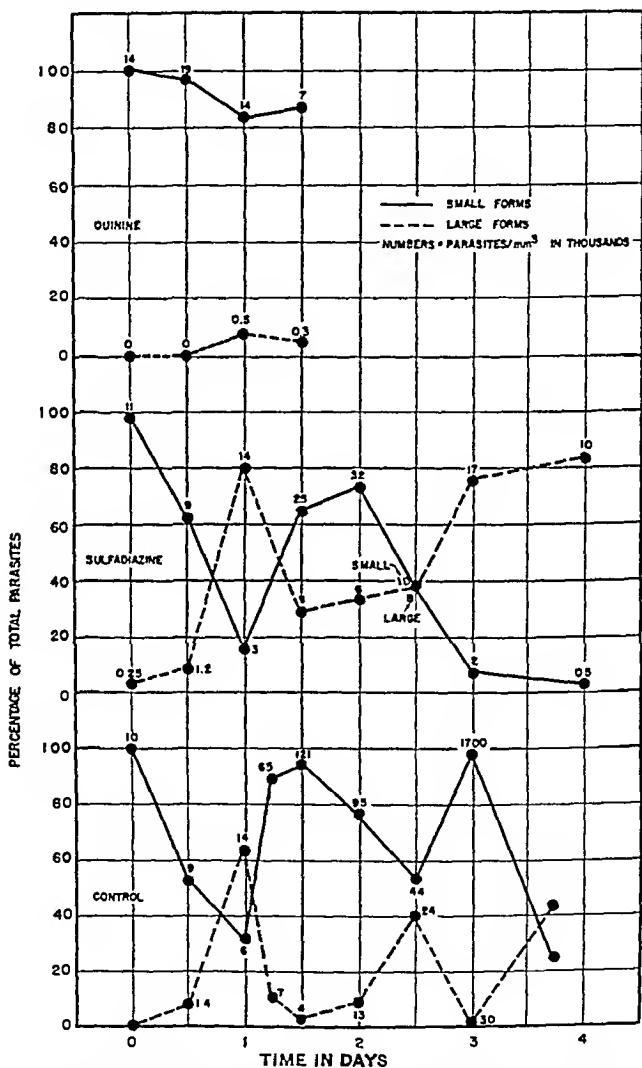


FIG. 2. THE EFFECT OF SULFADIAZINE AND QUININE ON THE GROWTH AND SEGMENTATION OF *P. GALLINACEUM*, AS SHOWN BY THE PERCENTAGE OF SMALL AND LARGE PARASITES. EACH VALUE REPRESENTS FIVE BIRDS

However, sulfadiazine partially inhibited the first treated generation. Growth was not markedly influenced during the first twelve hours, but by twenty-four

hours parasites with eight or more nuclei constituted 10 per cent of the untreated populations and only one per cent of the treated ones. Furthermore, during the first two days the treated parasites only increased four-fold in numbers, while the untreated ones increased fifteen-fold. In parasites treated for 36 hours, the average number of merozoites per segmenter was only 12.72 compared with 20.35 for untreated segmenters. (In both groups, 20 segmenters were counted in each of three birds. The standard deviations and the standard errors of the means were respectively 2.0 and 0.26 for the treated parasites, and 3.9 and 0.50 for the controls. The difference between the means is thirteen times its standard error and is therefore significant. On the contrary, the variation within each group was not statistically significant.) Low merozoite numbers account in part for the comparatively small increase following sulfadiazine treatment. Other explanations such as a reduction in the total number of segmenters, or in the viability of the merozoites cannot be evaluated with our data.

The second generation of sulfadiazine treated parasites was apparently unable to complete the cycle of segmentation and reinvasion (fig. 2, after $1\frac{1}{2}$ days). No parasites with more than four nuclei were seen after two days of treatment, and the percentage of small rings steadily declined. However, some growth occurred, since there is an increase in the number of large forms per mm^3 from 5,600 at 2 days to 16,700 at 3 days. The sulfadiazine treated parasites reached their population peak on the second day and declined to a sub-microscopic level by the fifth day, while with quinine, a sub-microscopic level was reached in only two days. The two drugs also differed in their morphological effects. With quinine many parasites were markedly abnormal by 24 hours, but not by 12 hours. Even after three days of sulfadiazine treatment, marked morphological changes were absent. However, by this time the stains appeared less brilliant, the nuclei were not as sharply defined, and the parasites became rounded.

The above experiment has been repeated with the same number of birds in the different groups, and the population curves completely confirm the original results. An additional experiment included five controls and five birds treated with only half the dosage of sulfadiazine, i.e., a 0.1 per cent drug diet giving blood levels of 3.5 mgm./ml. The initial inoculum contained many large multinucleate parasites, and within the first day there was a ten-fold increase in parasitemia in both the treated and the control birds. Under these conditions, therefore, sulfadiazine does not influence the invasion of erythrocytes by merozoites. The next cycle of population increase in the treated birds was only one-fourth that of the controls and thereafter parasitemia declined. Apparently the rate of inhibition is not altered markedly when the dosage of sulfadiazine is reduced from 0.2 per cent to 0.1 per cent in the diet.

In the latter experiment five birds were treated with a 0.1 per cent drug diet of 2-sulfanilamido-5-bromopyrimidine, "bromodiazine." This compound is more active than sulfadiazine on a blood level basis and is only partially reversed by para amino benzoic acid (unpublished data) which may indicate that its

mode of action differs from that of other sulfanilamides. Nevertheless, it is also slow acting, since differential parasite counts show that growth and segmentation are only partially inhibited even after two days of treatment. At this time, however, many parasites were morphologically abnormal, the segmenters had reduced numbers of merozoites, and parasitemia remained stationary.

We have also obtained some data on the rate of action of sulfadiazine and "bromodiazine" on the exo-erythrocytic stages from sporozoite-induced infections. Five-day old chicks were placed on drug diets and after one or two days inoculated intravenously with equal amounts of ground salivary glands from infected *Aedes aegypti* mosquitoes. At graded intervals after infection, the drug diets were replaced with the stock diet. The untreated controls all had more than 10 per cent of their erythrocytes parasitized on the eighth or ninth day after infection. In the treated groups, similar parasitemia was not found before 12 days, and some birds were still negative on the eighteenth to the nineteenth day. The latter were inoculated intravenously with infected blood at 24 and 32 days, respectively, in the "bromodiazine" and sulfadiazine groups. They all became blood positive indicating the absence of acquired immunity. This increases the presumption that the parasites of the initial sporozoite-induced infection were completely eradicated, and for our present purposes these birds are classified as "completely protected."

The data (table 1) show that with 0.1 per cent diet of sulfadiazine maximum effects, i.e., "complete protection" were obtained with four and one-half to six days but not with three days of treatment. With a 0.0125 per cent diet of "bromodiazine" maximum effects were not obtained even with five days of treatment. With a higher concentration of "bromodiazine," however, two to four days sufficed. The foregoing data suggest that sulfanilamides may not only act slowly against the erythrocytic forms, but also against the other stages of the malarial organism.

DISCUSSION. Our results on the rapid action of quinine in *Plasmodium gallinaceum* confirm those of Taliaferro et al. (7) in this infection, and those of Waletzky and Brown (8), and of Hewitt and Richardson (9) in *Plasmodium lophurae*. The results on the early treatment of sporozoite-induced infections with sulfadiazine are in line with those of Coggeshall et al. (10) who found that when treatment began nine days after infection more than five days were necessary to cause disappearance of the exo-erythrocytic forms.

The contrast in the speed of action of sulfadiazine and of quinine produces a characteristic effect in our routine blood-induced drug test. This test is very similar to that of Marshall et al. (3). In brief, 50- to 80-gram chicks are inoculated intravenously with about 20×10^6 parasitized cells, one day after the birds have been started on a drug diet. Alternating three-hour light and dark periods are used. Four days after inoculation, untreated controls have 50 to 80 per cent of the red blood cells parasitized. Concentrations of 0.05 per cent quinine in the diet suppress parasitemia to less than 0.1 per cent (approximately the initial level of infection) while the same concentrations of sulfadiazine result in a parasitemia of about 0.5 per cent. Higher levels of sulfadiazine do not give

lower counts. Our findings that quinine produces immediate stasis but that sulfadiazine does not prevent an initial increase in parasitemia, and that even after complete stasis elimination of the parasites proceeds slowly, seem to explain these results. Marshall and his co-workers (correspondence) have likewise observed that sulfanilamides do not depress parasitemia as much as quinine in their standard *Plasmodium lophurae* infections. From this and other observations they concluded that sulfanilamides act slowly in malaria.

Rose and Fox (11) suggested that bacteria "might be capable of undergoing a definite, limited number of cell divisions in the presence of any effective drug

TABLE 1

Activity of several sulfanilamides in sporozoite-induced infections of *Plasmodium gallinaceum*

DRUG	GM. DRUG/ 100 GM. DIET	BLOOD LEVEL	NO. OF DAYS OF TREATMENT AFTER IN- OCULATION	NO. COMPLETELY PROTECTED/ TOTAL NO.	INCUBATION PERIOD* OF BIRDS NOT PROTECTED
		mgm./ml.			
Sulfadiazine ^a	0.1	2.2	1.5	0/5	12
			3	1/5	16-18
			4.5	4/5	20
			6	5/5	-
Untreated controls ^a				0/8	8
"Bromodiazine" ^b	0.0125	1.25	2	0/4	15
			4	0/5	17
			5	3/5	19
	0.05	5.5	2	3/4	18
			4	4/4	
			5	4/4	
Untreated controls ^b				0/9	9

^a Treatment started 2 days before inoculation.

^b Treatment started 1 day before inoculation.

* Time in days from inoculation to peak parasitemia.

concentration" of sulfanilamides. They found that *Escherichia coli* underwent 3.6 to 6.85 divisions during treatment with bacteriostatic concentrations of sulfathiazole. Our results are not directly comparable, because division is schizogenous in the malarial organism. However, a comparison on the following basis seems permissible: The schizonts produced during the first one and one-half days in our test had an average of 12.7 merozoites, i.e., three to four divisions occurred, while subsequently no parasites with more than four nuclei were seen, i.e., not more than two further divisions took place. Therefore, the equivalent of five to six divisions occurred under our experimental conditions. The close numerical agreement with Rose and Fox's data is probably fortuitous, since

White and Himes in our laboratories (unpublished data) have found that *Escherichia coli* treated with sulfanilamides may divide fewer times under other conditions. However, the agreement in the order of magnitude still remains striking. It indicates that although the absolute time required for stasis with sulfanilamides may vary greatly, and is a function of organism size and growth rate, the relative "biological" time required for effective inhibition may be rather similar. If this is true for such widely different forms as bacteria and protozoa, it may also be valid within closely related groups. *Plasmodium gallinaceum*, for example, grows much more rapidly than *Plasmodium vivax* of man. (The former produces about 24 merozoites in 36 hours, the latter about 16 merozoites in 48 hours.) If drug susceptibility and other factors were equal, it may require more time to produce inhibition in *Plasmodium vivax* than in *Plasmodium gallinaceum*.

It seems likely that other chemotherapeutic agents will resemble the sulfanilamides in their relatively slow rate of action, particularly vitamin analogues, which act by interfering with the synthesis or utilization of essential substances. If the organism possesses stored reserves of such substances, complete inhibition may not occur until these are exhausted. This may explain the slow action of the sulfanilamides.

The activity of slowly-acting compounds against larger organisms may be overlooked in short-term tests, particularly under unfavorable *in vitro* conditions. For example, a number of parasitic protozoa can be "cultivated" *in vitro*, but frequently the rate of growth and multiplication is greatly reduced below that in the normal host. This reduced growth rate may not only make it more difficult to demonstrate inhibition, but may also render the organism less susceptible to inhibitory agents. Thus, White (12) found that lowering the temperature decreased the activity of sulfanilamide and sulfapyridine against streptococci *in vitro*.

Drugs which act quickly are essential for the therapy of the acute clinical phases of many diseases. In quinine and atabrin we have such antimalarial drugs, but compounds which could prevent or sterilize a malarial infection would be valuable. Such compounds need not act rapidly, since sulfadiazine can completely prevent a sporozoite-induced infection of *Plasmodium gallinaceum* (5) and sulfanilamide and several derivatives can sterilize an infection of *Plasmodium knowlesi* (13).

It would therefore seem desirable to prolong treatment and observation in both the laboratory and clinical testing of the protozoa, whenever it is possible. Such extended tests would not only be more sensitive to slowly acting compounds, but also to slight activity in new types of compounds. Although a compound with slight clinical activity would have no immediate practical significance, the demonstration of such activity may justify the synthesis of related compounds.

SUMMARY

1. a. The erythrocytic stages of the malarial parasite, *Plasmodium gallinaceum*, were treated continuously with large amounts of either sulfadiazine or

quinine and observed at frequent intervals to determine the rate of action of these drugs.

b. Quinine completely inhibited growth and development of the parasites almost immediately in four out of five birds and in the fifth bird did not permit development beyond the four-nucleate stage.

c. With sulfadiazine the first detectable retardation of growth occurred by 24 but not by 12 hours. Development of the first treated generation proceeded to segmentation, but the average number of merozoites produced per segmenter was only 12.7 as compared to 20.3 for the untreated controls. These merozoites invaded erythrocytes but did not develop beyond the four-nucleate stage before complete inhibition occurred.

d. With quinine the parasites never increased in number and by the second day had dropped below the initial count. With sulfadiazine there was a four-fold increase in parasitemia in the first two days, compared to fifteen-fold in the controls. The parasitemia declined to the initial level by the third or fourth day and dropped below it on the fifth day.

2. The compound, 2-sulfanilamido-5-bromopyrimidine, also acts slowly.

3. Sulfanilamides may also act slowly in sporozoite-induced infections since more than two days of treatment were required for maximum effects.

4. It is suggested that the length of time required for effective inhibition by sulfanilamides and other slowly acting compounds increases as the growth rate of the organism decreases. This may have practical implications in chemotherapeutic trials.

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FLAVICIN

II: AN ANTIBACTERIAL SUBSTANCE PRODUCED BY AN *ASPERGILLUS FLAVUS*¹

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Since the first report (1) describing an impure, penicillin-like, relatively non-toxic, antibacterial agent produced by a strain of *Aspergillus flavus*, others have reported similar findings with another strain of *Aspergillus flavus*. McKee and MacPhillamy (2) reported that a penicillin-like substance was obtained from deep cultures of White's strain of *Aspergillus flavus*, and later McKee, Rake and Houck (3) described some of its antibiotic properties and named it "flavacidin." *Aspergillus giganteus* has been reported to produce a penicillin-like substance (gigantic acid) (4). These antibiotics have not been described as pure chemical substances.

Our present report deals principally with progress in the production and purification of "flavacin". These studies have been restricted to the strain of *Aspergillus flavus* originally isolated by one of us (A. G.).

PRODUCTION AND PURIFICATION OF FLAVICIN. *Culturing of the mold* has been continued in the same manner as previously described (1) except that in the medium lactose has been substituted for dextrose. Activity of the culture filtrates has not been improved greatly, reaching an average maximum of 15-30 Staphylococcus Dilution Units (about 1 to 2 Oxford Units) per cc. in about seven days.

Assays generally are carried out as before by a serial dilution technique. The end-point is taken as intermediate between the highest dilution at which no visible growth occurs in all or nearly all of the tubes in 24 hours, and a higher dilution—usually 30 per cent higher—at which visible growth does occur in most of the tubes. We use two to ten tubes at each of the two critical dilutions depending upon the reliability desired for the particular assay. The strain of *Staphylococcus aureus* used has been checked frequently against a standard sample of calcium penicillin (135 Oxford U. per mg.) and 15-18 of our Staphylococcus Dilution Units (SU) are equivalent to one Oxford Unit. Occasionally activity has been estimated by the nitrite method (5). For the serial dilution assays we have made our sterile filtrates through small Corning glass bacteria filters² or specially made, modified (long-stem) Boerner filters (6) of stainless steel, of 5 cc. capacity, arranged for centrifugal filtration as shown in Figure 1, using standard 100 cc. brass cups. We have made several thousand of these filtrations (generally at 1000 to 1500 r.p.m., $r = 10.5$ cm.) with only a few breakages due to faulty test tubes.

For ease of removal and replacement of cotton plugs and of centrifugal filters and to

¹ Funds for carrying out this work were kindly supplied by the Mallinckrodt Chemical Works.

² These "UF" filters have about 0.3 cc. hold-up of water, and filter water at a rate of 1 cc. during 1-3 minutes when used in a centrifuge as described. Many filtrations have been made in succession without resterilizing and without apparent contamination, sometimes during periods of over a week; to do this it was necessary to remove nutrient materials by washing the filter with water before allowing it to stand until the next re-use.

strengthen the tubes we have found it advantageous to flare slightly and "bead" the open ends of all our pyrex culture tubes. For accuracy of pipetting we try to make the sterile

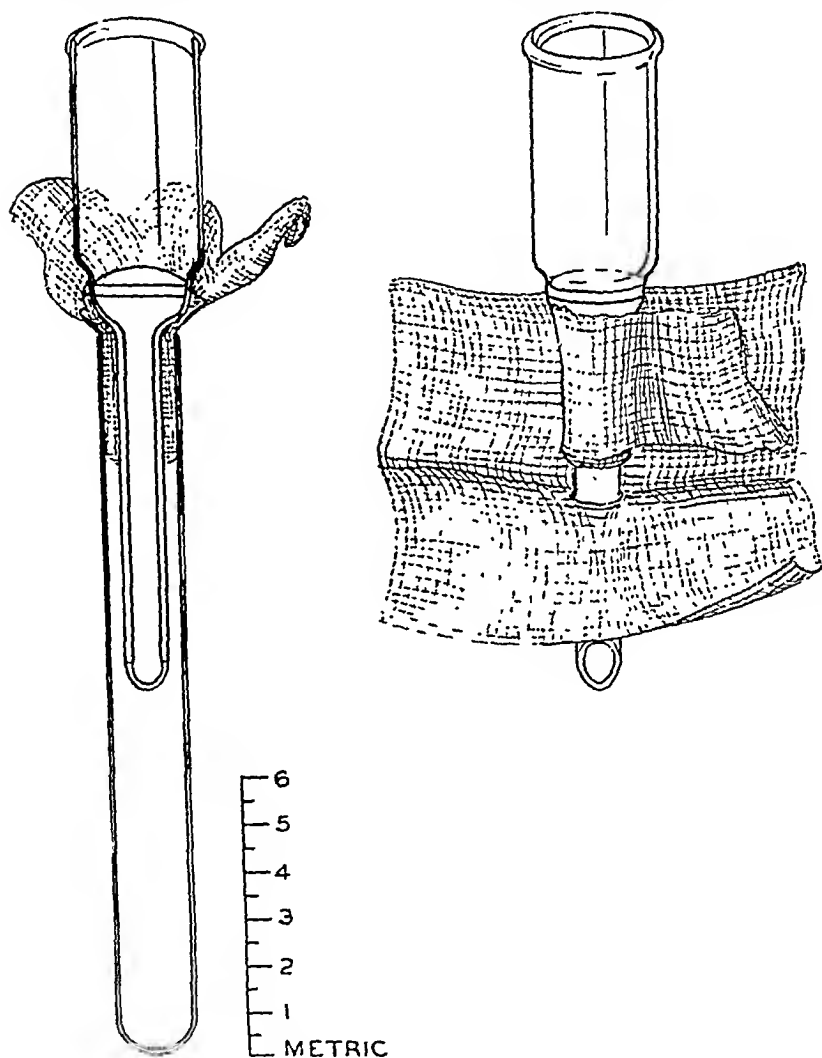


FIG. 1. ARRANGEMENT OF BACTERIA FILTER FOR CENTRIFUGAL FILTRATION

filtrates contain 10-50 SU per cc, and make appropriate dilutions in 5.0 cc. portions of sterile Difco nutrient broth; our graduated pipettes have been modified by making drawn out, bent, ground-off tips which are easily touched flush to the walls of the culture tubes.

All filtrates are stored on dry ice. When they are melted for making the dilutions they are kept ice-cold until refrozen.

Extraction of the culture fluid by means of a spray extraction column has been described in detail elsewhere (7). A modification of the apparatus there described which is sometimes advantageous consists of a jet head with 15 bores of 0.32 mm. diameter used in conjunction with a pressure head of about 80-100 cm. of culture fluid. In extracting batches of 80-100 liters of culture fluid we have used about $\frac{1}{3}$ this volume of *n*-butyl acetate or isopropyl acetate, and have extracted 3-liter portions of the acetate-extract successively with three 100 cc. portions of 0.5N aqueous NaHCO_3 . When the pH of the first bicarbonate falls to 7 it is retired and a fourth 100 cc. portion brought in, and so forth. For about 90 liters of culture fluid a total of 600-900 cc. of 0.5N bicarbonate is required. The yield of SU is usually about 50 per cent. This "crude extract" is kept on dry ice pending further studies. In such a non-sterile aqueous solution at 5°C. loss of anti-Staphylococcus activity may occur at a rate as high as 10 per cent per 24 hours.

(By immediately re-extracting the cold acid aqueous solution a further 10-15% recovery of SU sometimes can be obtained.)

The concentrated crude aqueous extract obtained as described above, like that obtained with isopropyl ether³ (1), is very toxic to mice. For convenience we describe the toxicity of a solution in relation to the anti-Staphylococcus activity by the ratio SU/MU, where SU is the number of Staphylococcus Dilution Units and MU is the number of Mouse Units. This "Mouse Unit" is defined as the LD₅₀ per gram of mouse. (In our estimations of the number of MU we have not attempted to attain an accuracy of greater than $\pm 25\%$.) The crude extracts generally represent about 50% yield from the cultures (where the culture fluid had 15-35 SU/cc.) and contain 1000-2000 SU per cc. and about 75-125 MU per cc. (SU/MU about 15-20) and have SU/mg.⁴ about 50-100.

With material from isopropyl ether extracts it was shown that much of this toxic material could be separated from the flavicin. About 10% of the activity was obtained with SU/MU > 60. The low yields and low activity were due to incomplete separation and to high losses by inactivation. When acetate-extracted material was examined by this procedure it was found that there was much toxic material which was not separated from the flavicin. In the light of subsequent findings this was due to the presence of a second toxic fraction in much larger amounts than in the ether extracts. It has been found that from the acetate extracted material two very different toxic fractions can be separated from the flavicin by means of systematic multiple fractional extractions with ethylene dichloride and aqueous solutions of controlled pH, or by chromatographic adsorption procedures. Application of these methods to crude extracts obtained with isopropyl ether also gave good recoveries of a

³ This ether is very dangerous on account of peroxide formation. Originally and before each re-use the peroxide is removed by shaking with excess aqueous SO_2 ; the SO_2 is then removed by shaking the ether with excess aqueous NaHCO_3 . The ether may be redistilled before removal of the SO_2 .

⁴ The determination of the organic solute in mg. is carried out by extracting a small portion of the aqueous solution at pH 2-3 with two or three volumes of isopropyl acetate (IPA), evaporating this solvent, and weighing the residue. This process results in losses of about 80% of the activity within 30-60 minutes at 25°. If ethylene dichloride (ED) is used instead of isopropyl acetate 80-100% of the activity may be recovered when the residue is redissolved in aqueous NaHCO_3 .

relatively non-toxic antibiotic which was apparently identical to the material from acetate extracts.

Purification of flavicin by systematic multiple fractional extractions. The multiple fractional extraction method is discussed by Hunter and Nash (8), Morton (9), Craig (10, 11), and others. The following is a description of the development and application of this method to our problem.

Our early fractionation results were obscured by high losses of SU, which occurred principally in the aqueous solutions of low pH and to a lesser extent in the organic solvents. These losses were minimized by working rapidly at the lowest practicable temperature, 0-5°C. It was found advantageous to complete the extractions, get the flavicin back into aqueous solution at pH 7-8 and put this on dry ice as soon as possible.

It was possible to calculate, from data obtained from experiments involving successive partial extractions and multiple fractional extractions, the approximate distribution coefficients for two different toxic fractions and for flavicin. Diagrams 1 and 2 show the details of some of these experiments and some of the data for the following calculations of distribution coefficients:

(1) a toxic acidic fraction (A) very soluble in water (diagram 1, ED III and AqI₃)

$$\frac{\text{MU of A per cc. Ethylene Dichloride}}{\text{MU of A per cc. water}} = \frac{6}{44} = 0.13 \text{ where the water contains sodium phosphate-} \text{H}_2\text{PO}_4 \text{ mixture (0.3M) and has pH 2.5}$$

(2) a toxic acidic fraction (B) very soluble in Ethylene Dichloride (ED) (Diagram 1, ED I and ED II-ED III; Diagram 2)

$$\frac{\text{MU of B per cc. ED}}{\text{MU of B per cc. water}} = \frac{200}{1} = \text{about 200 where the water contains phosphate (0.3M) or NaCl (2\%)} \text{ and the pH is 2.5-3.5 (H}_2\text{PO}_4\text{)}$$

(3) flavicin (SU) (Diagram 1, ED I and AqI₃-ED I) (also Diagram 3)

$$\frac{\text{SU/cc. ED}}{\text{SU/cc. H}_2\text{O}} = \frac{1850}{380} = 5 \text{ where the water contains phosphate (0.3M) and the pH is 2.5 (H}_2\text{PO}_4\text{)}$$

$$= 1 \text{ where the water contains NaCl (2\%) and the pH is 3.5 (trace of H}_2\text{PO}_4\text{)}$$

For a less crude fraction (300 SU/mg)

$$\frac{\text{SU/cc. ED}}{\text{SU/cc. H}_2\text{O}} = \frac{45}{375} = 0.12 \text{ where water contains 2\% NaCl and the pH is 4.5 (obtained by adding sufficient NaHCO}_3\text{)}$$

$$\frac{\text{SU/cc. IPA}}{\text{SU/cc. H}_2\text{O}} = \frac{900}{3000} = 0.3 \text{ where water contains 2\% NaCl and the pH is 4.5 (obtained by adding sufficient NaHCO}_3\text{)}$$

These rough distribution coefficients made possible an approximation of the volume ratios of the solvents which would give a reasonable separation.

Although it is possible to carry out the separations in one continuous series of extractions, it is much easier (on a small laboratory scale) first to concentrate and separate most of the flavicin + toxic fraction B from most of the toxic fraction A; this allows the subsequent extractions to be carried out with much smaller volumes than otherwise would be necessary.

The ratios of the volumes of the two solvents which will give a "symmetrical separation" of the SU and A can be calculated from the appropriate distribution coefficients.*

$$\frac{\text{Volume ED}}{\text{Volume water}} = \sqrt{\frac{1}{5 \times 13}} = 1.2$$

* For more crude specimens of flavicin this coefficient is near 1. This evidence that a second "flavicin" exists in the crude is more clearly shown in Diagram 1 by the high value for SU AqI₄ (Also in Diagram 3).

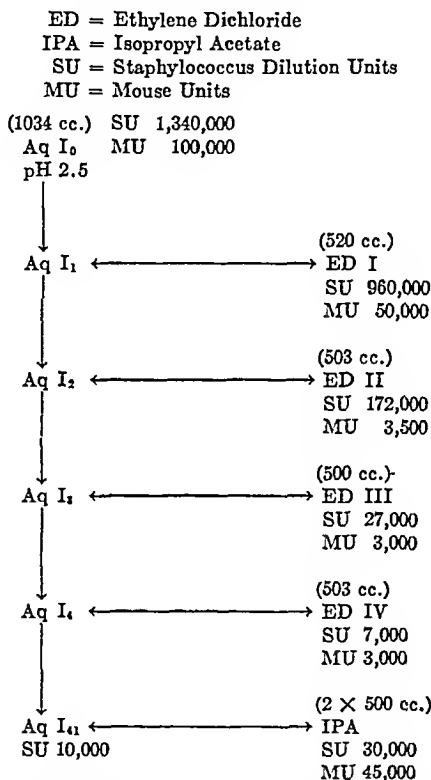
* A general equation was derived some six years ago by Thomas C. Butler: $\frac{V_1}{V_2} = \sqrt{\frac{1}{C_x C_y}}$ where C_x and C_y are distribution coefficients of substances x and y between solvents 1 and 2. This volume ratio gives a "symmetrical" separation.

With this volume ratio it is easy to calculate from the distribution coefficients that SU and A should be separated to an extent of about 95% (with all the B in the "ED pool") by the following procedure: (See Diagram 3)

This initial separation (and concentration) is generally carried out with batches of 1 to 2 liters of crude extract containing 1 to 4 millions of SU (about 20-40 grams of acidic organic

DIAGRAM 1

Extraction of "Staphylococcus Units" and "Mouse Units" from a crude extract of A. flavus culture



The cold acidified "crude extract" (Aq I₀) was extracted in succession with four portions of ED, then exhausted by two portions of IPA, as shown. These extracts were separately exhaustively extracted with several small portions of 0.2N NaHCO₃. The latter were combined in each case. Results of the assays are shown. For further discussion see text.

solute from about 100-200 liters of *Aspergillus flavus* culture fluid). This aqueous solution is acidified to pH 2.5-2.0 with H₂PO₄ and extracted as rapidly as possible with two portions of ethylene dichloride each 1.2 times the volume of the aqueous solution (or with three 0.6 volumes of ED if the volume of aqueous solution is large). These ED extracts are kept separate and are extracted rapidly in order and in succession with a volume of 2% aqueous

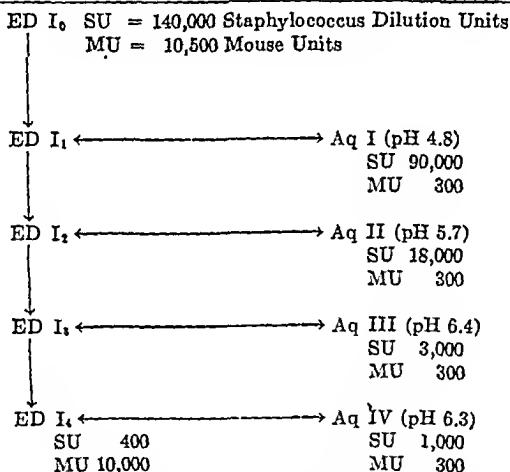
NaCl equal to the original volume of acidified aqueous solution (or 0.5 this volume if 0.6 volumes of ED were used). A pH of 2.5-2.0 after each of the extractions is maintained by adding H_3PO_4 if necessary.

The ED's are combined and extracted with 50 cc. of 0.40 N, then with 50 cc. of 0.1N, aqueous $NaHCO_3$ (shaking 3 minutes each time). The pH of each of these aqueous extracts should be 7-8. They are combined ("ED pool") and contain 75-90% of the SU, approximately half the toxicity and 10-15% of the weight of organic solute of the crude extract. Some solute and MU but no SU are left in the ethylene dichloride. If desired the organic acids in the aqueous acid solutions can be extracted by isopropyl acetate (3 equal volumes), then into N NaOH solution in sufficient volume to give at equilibrium pH 7.5-8.5. The

DIAGRAM 2

A crude aqueous solution of flavicin and toxic fraction B was acidified to pH 2.5 and extracted with two equal volumes of Ethylene Dichloride (ED)

These were combined (48 cc.) and extracted with 250 cc. portions of 2% aqueous NaCl as follows:



The cold solution ED I₀ was extracted in succession with four portions of aqueous 2% sodium chloride solution, as indicated; after this the ED I₄ was shaken with 3×10 cc. of 0.1N $NaHCO_3$. The solute was concentrated from the aqueous phases by extraction with IPA, then taken into small volumes of 0.2N $NaHCO_3$. The toxic fraction B is mostly retained by the ED, while the flavicin is extracted by the salt solution. For further discussion see text.

"Aq pool" contains about 10% of the SU, half the toxicity and 80-85% of the weight of organic solute of the crude extract. The aqueous alkaline extracts called "ED pool" and "Aq pool" are kept on dry ice pending further treatment.

Calculations based on the distribution coefficients show that SU and toxic fraction B can be separated by a simple multiple fractional extraction using two portions of ED each 1.0 volume, and two portions of aqueous 2% NaCl, pH 3.5, each of about 14 volumes. If the volumes of ED must be larger than about 100 cc. or if more than 100,000 SU are present it is desirable to limit the

DIAGRAM 3

Purification of flavicin by multiple fractional extractions

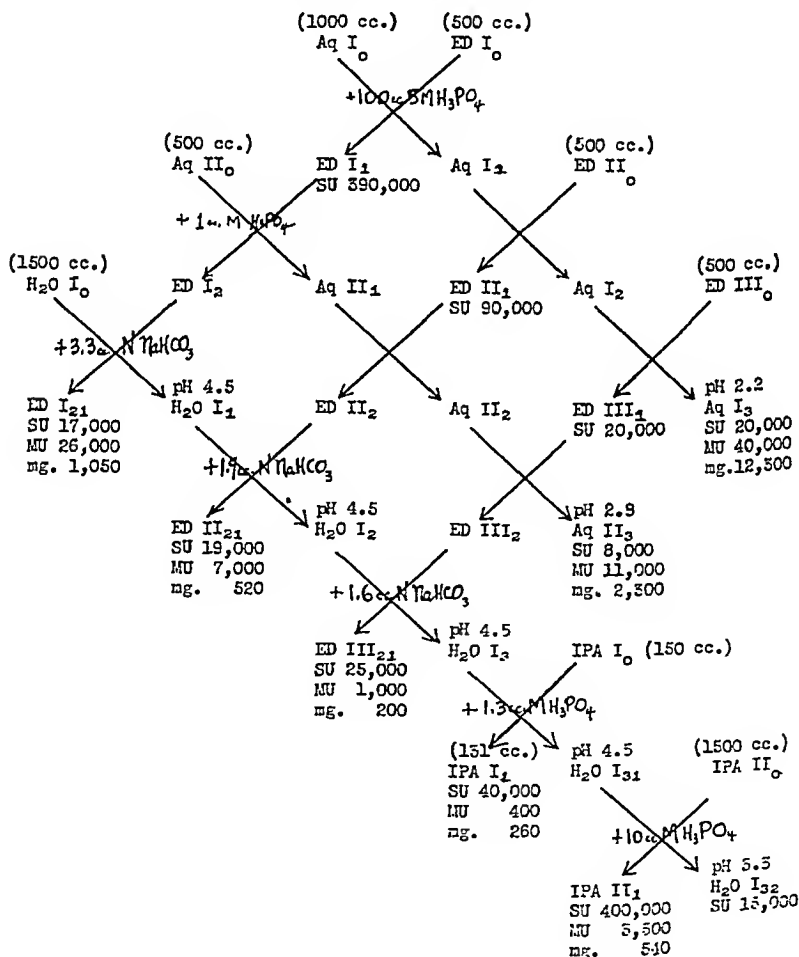
Crude Extract (25% yield from cultures)

SU 0.55×10^4 —Staphylococcus Dilution Units

MU 130,000—Mouse Units

mg. 19,500—milligrams

This representation of the distributions of SU, MU and mg. gives a view of the nature of the crude extract. It is shown by other experience, and is indicated by these data that the MU retained in the main fraction (IPA II₁) would have been almost completely removed if one or two more "Aq's" (low pH) and possibly one more "ED" had been used. (See Diagram 4.)



volume of aqueous 2% NaCl to about 1500 cc. and maintain a pH of 4.5 with appropriate additions of NaHCO_3 . It has been found advantageous (in obtaining a less impure product) to combine this series of extractions with a repetition of the first series.

The modified procedure is carried out at 0-8°C. as follows (for example see diagram 4, page 270).

About 100-600 cc. of aqueous solution "ED pool" containing about 10,000 to 50,000 SU/cc. (about 20-100 mg./cc.) is acidified to pH 2.5-2 with H_3PO_4 and extracted as quickly as possible with three portions of ethylene dichloride each equal in volume to the aqueous solution. The three ED's are extracted in order and in succession with an equal volume of 2% aqueous NaCl containing about 5 millimols H_3PO_4 per liter (pH 2.5-2.0). It is advantageous to carry through in like manner a third (and possibly a fourth, etc.) acid aqueous solution. If it is desired to recover only flavicin these Aq's are discarded. Some activity and toxicity can be recovered from them by extraction with IPA, then by extraction of the latter with bicarbonate.

The three ED's are now shaken in order and in succession with two 1500 cc. portions of 2% aqueous NaCl. If (for convenience) the volume ratio is $> 1/14$ (i.e., $1/7$ to $1/2$) the pH of these aqueous layers ("H₂O I" and "H₂O II") is kept near 4.5 by adding portions of N NaHCO_3 as needed to give this pH value after equilibrating with each ED. Finally these aqueous solutions usually contain respectively 80-85% and 10-15% of the original SU. The three ED's contain practically 100% of the toxic fraction B and less than 2% of the SU of the "ED pool."

The antibiotic material is obtainable by acidifying H₂O I, and H₂O II, to pH 3-2 with H_3PO_4 and extracting successively (quickly) with two 1500 cc. portions of IPA. These may be kept separate if desired. The IPA gives up the flavicin quantitatively to three 20 cc. portions of .2N NaHCO_3 (shake each for 3 minutes, pH 7-8, 30-60 minutes for each separation). The antibiotic so obtained usually has SU/mg. 1500-2500, SU/MU > 1000 (mice show no symptoms for 24 hours after receiving intraperitoneally 500 mg./kg.).

It has been found that the SU/mg. is raised considerably if instead of stopping the multiple fractional extraction process at the stage H₂O I, and H₂O II, a new fractionation is introduced by extracting these in succession with several small (0.05 to 0.1) volumes of IPA while the pH of both aqueous phases is relatively high. It is generally necessary to add small portions of M H_3PO_4 to keep the pH values down to 4.5 after equilibration with each IPA. If the pH of the H₂O phases falls much below 4.5 these acetates take out more SU than is desirable. After carrying through "IPA I₂," "IPA II₂," and "IPA III₂," the main fraction of SU is obtained as described above.

In diagram 4 a typical multiple fractional extraction process as described above is illustrated. The total time required for these operations is 4-8 hours when all fractions are saved. "Aq I₀" was made up of eight "ED-pools," each of which was obtained as described in the foregoing. The total volume of *Aspergillus flavus* culture fluid represented is approximately 700 liters. The main fraction "IPA IV₂" has 3000 SU/mg. (200 Oxford U./mg.), and the fraction "IPA V₂" has 2000 SU/mg. These were combined and again put through the series of extractions as shown in diagram 4. The main fraction of 4.5×10^4 SU weighed 900 mg. (5000 SU/mg.). Two more such treatments (using only "IPA IV₂" each time) gave 2.2×10^4 SU and 180 mg. (12,000 SU/mg.). The total recovery of SU in all fractions was 85-100% each time the material was processed. When the batch of 2.2×10^4 SU was put through the process the main fraction (IPA IV₂) weighed 107 mg. and had 12,000 SU/mg. The sum

DIAGRAM 4

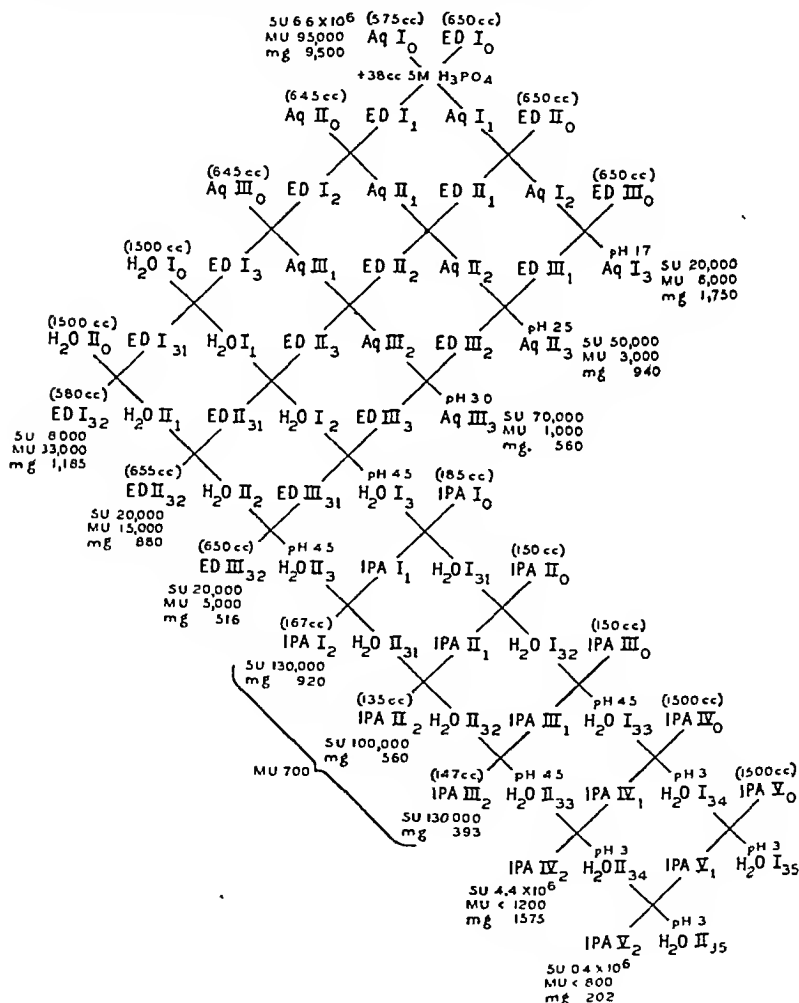
Purification of flavicin by systematic multiple fractional extractions

SU = Staphylococcus dilution units

MU = Mouse units

mg. = Milligrams

The material in "Aq I₀" is a mixture of many small batches of "ED pools" from which most of the toxic fraction A has been removed. Details not shown are described in the experimental part. The overall loss of MU may be partly due to decomposition, and partly to errors of assay. Loss of SU is largely due to decomposition (chemical inactivation)—this amounts to approximately 15-20 per cent in this case.



of the SU in all fractions was, however, only 1.85×10^5 (84%), and thus it appears that the apparent constancy of the SU/mg. is not necessarily indicative of purity; the loss possibly just balanced what otherwise would have been an appreciable increase in SU/mg.

The problem of the further purification of flavicin is being studied.

The purification of flavicin or penicillin obtained from urine can be quickly carried to 4000-8000 SU/mg. by the procedure as described. Over one million Oxford Units of crude material can be handled at once in the volumes shown.

The actual value of SU/mg. attainable by a given number of fractional extractions varies, of course, with the relative amount of inactive material originally present. Cultures of relatively low activity and/or relatively low yields (high losses by decomposition may lead to the formation of closely related inactive substances difficult to separate from the flavicin) during the manipulations give relatively low values to SU/mg. at any given stage. Our many efforts to raise the initial yield (extraction from the culture) have failed and in general 50% is the best we can attain. Our early report (1) of yields of 75-100% it now seems was based partly upon relatively less accurate assays, but was also associated with cultures of somewhat lower activity.

Through our many experiments there runs strong evidence that there is more than one penicillin-like substance (one extremely labile, the other moderately labile) present in our cultures. The facts that suggest this are:

(1) Loss of activity in specimens of our usual culture fluid (about 30 SU/cc.) at 0°C. and pH 3-4 occurs at a very high rate until 25-50% loss is reached (about 120 seconds), then at a relatively low rate. (Certain other specimens of culture fluid lose activity much more slowly.)

(2) In the fractionation of "crude extracts" containing 50% yields from culture fluid of activity 35 SU/cc. there is a relatively large amount of active material (perhaps 15-20%) in the first aqueous phase after the several ED extractions. Subsequent fractionation of the "ED pool" does not show this fraction (i.e., active material with relatively high distribution into the aqueous phase at pH 2.5). Fractionation of crude extracts containing only 25% yields from the cultures give little of this fraction (diagram 3). Old cultures (15 days) of low activity probably contain relatively more of the more stable kind of flavicin, which would lead to high yields in the crude extracts. Similarly, young cultures (6-8 days) may contain relatively more of the less stable kind of flavicin, and this may be associated with the low yields in our crude extracts. Both age and composition of medium probably influence the relative amounts of these substances (and others, such as aspergillilic acid (3)) in the cultures. We have not made a systematic analysis of these factors.

There is an analogy between this behavior and that of crude extracts from shallow cultures of *Penicillium notatum* (Fleming) (American Type Culture Collection strain), grown on the Czapek-Dox-corn-steep medium. Here the yield in the initial extraction is higher (about 80%) but there is some 10-50 times as much activity present in the cultures (the total amount of organic acids in the crude extracts is approximately the same as in those from the cultures

of *A. flavus*). Fractionation of the crude extract by the extraction process separates two active fractions rather sharply, one accumulating in the ethylene dichloride and the other in the aqueous solution of low pH. Our evidence indicates that the latter of these is the more labile under these conditions and amounts to about 30-40% of the activity of our crude extracts. Further fractionation of the former fraction gives nearly quantitative recoveries (relatively stable) and high SU/mg. ($> 8,000$). Similar pictures have been obtained in the fractionation of crude penicillin from urine of patients.

Discussion of the multiple fractional extraction method. This method has been applied in this laboratory to the separation and identification of alurate and narconumal in crude extracts of urine (12) to the separation of several degradation products of evipal found in extracts of urine (13), and to other problems. If one is searching for known compounds in unknown mixtures one can make use of appropriate distribution coefficients to calculate separations by fractional extraction. Thus in the case of alurate and narconumal it was possible to calculate a quantitative separation which when carried out gave two narcotically active fractions. From one of these alurate was crystallized. The distribution calculations indicated that the narcotic activity of the other fraction was not due to alurate but rather to narconumal, although it could not be crystallized. (It is to be remembered, of course, that other solutes may alter the distributions; but this can usually be minimized by working with dilute solutions.)

If one is attempting to isolate new substances which can be assayed biologically, appropriate distribution coefficients can often be found by means of such assays. To separate a substance from a mixture it is very advantageous but not always necessary to know the distribution coefficients of other components of the mixture. A considerable separation can often be achieved simply by adjusting the volumes of two appropriate immiscible solvents so that after the first extraction about 25% of the material is in one or the other solvent, and after a number of extractions most of the desired substance has accumulated in one of the solvents; then starting all over again with a different volume ratio such that the desired substance accumulates in the *other* solvent. If a mixture consists of several very closely related substances, any slight differences in distribution can be greatly multiplied by carrying the fractional extraction process to an advanced stage—say 50 to 500 extractions. In some such cases we have observed that certain intermediate fractions may show properties distinctly different from the fractions on either side. These fractions can be isolated and studied further (for example, re-crystallized). For application of this procedure to analytical purposes see Craig (10, 11).

The general method is adaptable as a fine analytical tool for demonstration of chemical homogeneity, or lack thereof, and for obtaining evidence as to the identity of known substances occurring in unknown mixtures (Craig, l.c.). It is often possible to do these things without ever isolating the chemical substance(s) in question. For example, the application of the method described to crude penicillin shows immediately that—as obtained from shallow cultures

of *P. notatum* on Czapek-Dox medium containing corn steep water—at least two active “penicillin-like” substances are present.

It should be possible to produce flavicin or penicillin of relatively high purity by adaptation of the discontinuous multiple fractional extraction method to a series of continuous counter-current extractions.

Purification of flavicin by chromatographic adsorption. It was found that chromatographic procedures employing impregnated silica gel (14) (15) (16) and acidified alumina (17–19) in succession, effect a remarkable separation of the antibacterial material from most of the other substances present in the crude extracts of our *Aspergillus flavus* cultures. It was found advantageous to use an IPA extract of the “ED pool” material described elsewhere as a starting point instead of beginning directly with an IPA solution of the crude extract material. The latter point has been used, and with good results, but the great reduction in solid materials with little attendant loss in antibiotic activity accomplished in obtaining the “ED pool” makes this the more logical place to begin. The earliest work was done with the IPA solution of the crude extract and from this solution it was possible to separate in considerable amounts two different toxic materials from the flavicin. One of these toxic materials is largely removed in making the “ED pool.”

The silica gel used was prepared from commercial water glass (Merck) according to the method of Martin and Synge (14) omitting methyl orange and using an external indicator. After drying it was screened so that 75% was 60–100 mesh size. The alumina used was “Fisher Adsorption Alumina” (Fisher Scientific Company).

Impregnated silica-gel column. The “ED pool” containing 800,000–1,500,000 SU is acidified to pH 2.5 and the acidic materials are taken into approximately 50 cc. of IPA (two 20 cc. and one 10 cc. portions). The IPA solution is then put through an impregnated silica gel column (15, 16). The silica is packed into a tube having an inside diameter of 15 mm.; 1 gm. of silica produces a column height of about 2.1 cm. Usually a total of 18 gm. of silica was used. The column holdup is determined beforehand by passing IPA saturated with water through it.

Our best results, as described below, were obtained by impregnating the silica with our modification of a method which has not yet been released for publication by others.

When the IPA solution has all entered the column the latter is then developed with 270 cc. of IPA saturated with water. Three percolates are taken. The first percolate has a volume equal to the holdup of the column plus the volume of the IPA solution of the extract. No color appears in the percolate until the holdup is exceeded. The second percolate has a volume of 35 cc. The third percolate contains all the rest of the IPA solution coming through the column, 217–225 cc. The column at this stage has a dark brown zone at the top extending downwards some 5–7 cm. Below this zone the color tends to shade off to a light brown and then to a dirty yellow and finally to a uniform yellow color. The column is extruded and broken into three segments. The bottom and middle segments are 15 cm. each, while the top one is 7 to 10 cm., depending upon the original length of the column. This latter fraction is the one containing the dark brown zone.

The percolates are extracted with 1×80 cc. of 0.4N NaHCO_3 and 2×80 cc. of 0.2N NaHCO_3 . The pH is 7–8 after each extraction.

The segments, or fractions, of the column are placed in beakers containing 10 cc. of N NaHCO_3 and are allowed to stand for 5–10 min. At the end of this time the contents of the

beakers are transferred to small Buchner filter funnels and the NaHCO_3 solution removed by filtration. The silica is washed on the filter four times with sufficient water to make a fine slurry. Washings and NaHCO_3 extracts are combined.

The first percolate contains only a small percentage of antibiotic material but a major portion of toxic material (fraction B). The second percolate contains a fair amount of the toxic material and slightly more antibiotic material than the first. The third percolate contains the major portion of the antibiotic material and little, if any, of the toxic material. (It may contain some if the "ED pool" is not used as a starting point). The segments of the column usually do not contain any appreciable amount of antibiotic material—occasionally the bottom segment contains a small amount. The top segment contains a fair amount of toxic material (probably fraction "A") with lesser amounts in the other two segments).

The distribution of solid materials is especially interesting. Over 50% of the solids originally present in the ED pool are collected in the first percolate and the top segment of the column with approximately equal distribution between these two. The third percolate contains some 10% of the total solids and 85-100% of the SU originally present. It possesses a slight yellow color.

The material in the third percolate has on the average an activity of about 150 Oxford Units per milligram.

Acid alumina column. An alumina column is prepared by placing 50 gm. of Fisher's Adsorption Alumina in a tube having an inside diameter of 15 mm. This produces a column 38 cm. in height. Fifty cc. of 2.5N acid (HCl or H_2SO_4) is then allowed to run through. Afterwards the column is washed with water until the washings coming through have pH 4.0-4.1 (17-19).

When the above pH has been reached the 24.0 cc. of NaHCO_3 extract of percolate three above is put through the column. The column is then developed and eluted with 100 cc. of distilled water. Three percolates are taken. The first percolate has a volume of 36 cc.—column holdup plus 2.0 cc. The second percolate has a volume of 50 cc. and is a light straw color. Color begins to appear in the first few cc. of this fraction. The last 5-10 cc. of this second percolate has little color. The third percolate is made up of the rest of the aqueous solution coming through the column—usually about 45 cc. Two cc. of N NaHCO_3 is added to each of the three percolates to bring the pH to 7-8. The column at the end of elution shows only a dark brown zone at the top.

The column can be washed up as one segment in the same way as a silica gel column fraction is treated, but this is usually not worthwhile as it contains no appreciable antibiotic activity.

The first and third percolates and the extract of the column do not show any antibiotic activity worthy of note. The second percolate contains almost all the original activity. The activity per milligram of this material is 300-350 Oxford Units.

Repeated use of the above two columns on the partially purified flavicin yields a product of greater purity. To date the highest degree of purity we have attained is approximately 800 Oxford Units per milligram.

PROPERTIES OF THE TOXIC FRACTIONS. The bicarbonate solutions ("crude extracts") obtained from isopropyl ether extracts of the culture fluid gave on acidification a precipitate (1): this contained much of the toxicity and some of the antibiotic activity. Purification of this material has not been pursued beyond the demonstration that it shows an antibiotic activity which is not due to flavicin as an impurity. It is soluble in ether and ethylene dichloride, insoluble in water, readily soluble in cold aqueous NaHCO_3 (0.2N). It has 25 SU/mg. and 40 MU/mg. and the ratio of SU/mg. to *B. subtilis* U/mg. is significantly different from that of flavicin. These properties are probably due only in small

part if at all to "Aspergillie Acid." The toxic fraction B obtained from the acetate extracts has not been purified. In the crude state this has some slight antibiotic activity apparently not due to flavicin and LD50 for mice of about 25 mg./kg. The toxic fraction A in the crude state has LD50 for mice of 250 mg./kg. Several recrystallizations of this material (the fraction Aq I₃ represented in diagram 3) from IPA and from benzene, alternately, have led to a 10% yield of an apparently pure colorless substance, having m.p. 67.5–68.5°C. corr. Electrometric titration with NaOH shows two apparent ionization constants: pK'_1 3.7 and pK'_2 9.1. The first break in the titration curve indicates a neutralization equivalent of 124. This substance seems to have no antibiotic activity.

Further study of these materials is under way.

PROPERTIES OF FLAVICIN. *Activity of flavicin in vivo.* Our earlier report (1) described the protection of mice against certainly-fatal doses of pneumococci by our then very crude flavicin. With slightly larger amounts of much less crude material (150 Oxford U./mg.) we have with single doses of flavicin completely protected mice against certainly-fatal doses of pneumococci. Flavicin of this, or somewhat higher purity produces no apparent toxic effects in mice when given intraperitoneally in doses of 1 gm./kg.

A sensitive test for the irritating properties of drugs is the effect after intrathecal injection on the cerebro-spinal fluid cell count. Dr. Cobb Pilcher has kindly tested a sample of flavicin (150 Oxford U./mg.) by the technique which he and his co-workers have developed in dogs (20) and found that single intrathecal injections of 500 Oxford U. in 1 cc. of isotonic solution at pH 7.4 in each of 3 dogs caused a rise in the cell count of no more than occurred in controls from the injection of saline.

Clinical use of flavicin. Only a few months ago when penicillin was scarce many opportunities arose for the use of flavicin in human patients. Unfortunately far too little was available for a single satisfactory course of treatment. In one case of severe *Staphylococcus aureus* septicemia with multiple abscesses throughout the body some 20,000 Oxford Units (300 mg.) of flavicin (the total available supply) was given in several doses during 24 hours, and the number of Staphylococci in the blood fell from over 200 to about 6 per cubic mm. The next day penicillin arrived and the patient was continued on this drug. Another patient with a spinal abscess and septicemia due to a *Staphylococcus aureus* was given some 15,000 Oxford Units of flavicin in three doses during 24 hours, and the number of Staphylococci in the blood was reduced from some 200 to about 3 per cubic mm. This patient eventually recovered through treatment with much larger amounts of penicillin, but the clinical opinion was that death would have occurred within 24 hours if flavicin had not been given.

Comparison of flavicin and penicillin. Because of their great similarity in physico-chemical and antibiotic properties we have made some careful comparisons of our "flavicin" with "penicillin" obtained from shallow cultures of *Penicillium notatum* (Fleming) grown on the same medium and purified to the same degree (500 Oxford U./mg.) by the extraction processes. The purification

thus eliminated from *both* specimens the more labile active substance(s) which were present originally, the active substance(s) which have a relatively low distribution ratio between ethylene dichloride and aqueous salt solution of pH 2.5 and any active substances which have a high distribution ratio between ethylene dichloride and aqueous salt solution at pH 4.5 (for example, aspergillilic acid-like substances).

These specimens of flavicin and penicillin could not be certainly differentiated by measurements of distribution coefficients between ethylene dichloride and aqueous 2% NaCl at pH 2.90 or at pH 4.80, or between isopropyl acetate and aqueous 2% NaCl at pH 4.2. The accuracy of these comparisons was limited by the error of our assays, about $\pm 15\%$. If this error could be reduced sufficiently this method of comparison of distributions between immiscible solvents would be excellent for the demonstration of probable identity or lack of identity of these chemical substances—without isolating them (cf. Craig, l.c.).

No significant difference could be detected between "penicillin" and "flavicin" when they were examined by the chromatographic procedures outlined previously.

These specimens and a crude specimen of the more labile and more water-soluble "penicillin" also showed the same antibacterial spectrum when tested against *Staphylococcus aureus*, *Streptococcus hemolyticus*, *B. anthracis* and *B. subtilis*, and all were relatively inactive against *Pseudomonas pyocyaneus* and *E. coli*. Because of the great labor otherwise involved these comparisons were not done with sufficient accuracy to show whether differences smaller than 25% exist.

It is then clear that flavicin and penicillin obtained and purified as described above are very closely related, possibly identical, substances. Comparison of pure specimens by more accurate methods is required to demonstrate whether they are identical.

In their recent paper (3) McKee, Rake and Houck describe the production, by various strains of *Aspergillus flavus* grown in submerged cultures, of two antibiotic substances, the aspergillilic acid of White (21) (3) and flavicidin, a penicillin-like substance. In these cultures the one or the other type of antibiotic activity predominated, according to the nature of the medium.

Although the flavicidin described was not pure (maximum activity mentioned, 214 Oxford U./mg.) its chemical and antibacterial properties and low toxicity indicate that it is very similar to, or identical with, "flavicin" and "penicillin". The differentiation or identification of the two or more kinds of "flavicin," the two or more kinds of "penicillin," and "flavicidin" (more than one kind?) will require study of more highly purified specimens.

SUMMARY

The application of systematic multiple fractional extraction methods and of chromatographic adsorption procedures to the further purification of flavicin is described. Flavicin having activity of 12,000 *Staphylococcus* Dilution Units per mg. (800 Oxford Units/mg.) has been obtained by both methods. This

material is probably not yet pure. The toxicity in mice of specimens of flavicin having 200-300 Oxford U./mg. is so low that 1000 mg./kg. intraperitoneally produce almost no symptoms. Some pharmacological effects of flavicin are described, including beneficial results in two human patients suffering from *Staphylococcus septicemia*.

Flavicin and penicillin, both purified by the same method to an activity of 500 Oxford Units per mg., are very similar in their chemical and antibiotic properties. Further studies of more nearly pure specimens will be required to demonstrate whether they are identical or not.

We are greatly indebted to Miss Rebecca Taylor and to Miss Charlotte Ward for both chemical and bacteriological assistance.

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thus eliminated from both specimens the more labile active substance(s) which were present originally, the active substance(s) which have a relatively low distribution ratio between ethylene dichloride and aqueous salt solution of pH 2.5 and any active substances which have a high distribution ratio between ethylene dichloride and aqueous salt solution at pH 4.5 (for example, aspergillie acid-like substances).

These specimens of flavicin and penicillin could not be certainly differentiated by measurements of distribution coefficients between ethylene dichloride and aqueous 2% NaCl at pH 2.90 or at pH 4.80, or between isopropyl acetate and aqueous 2% NaCl at pH 4.2. The accuracy of these comparisons was limited by the error of our assays, about $\pm 15\%$. If this error could be reduced sufficiently this method of comparison of distributions between immiscible solvents would be excellent for the demonstration of probable identity or lack of identity of these chemical substances—without isolating them (cf. Craig, l.c.).

No significant difference could be detected between "penicillin" and "flavicin" when they were examined by the chromatographic procedures outlined previously.

These specimens and a crude specimen of the more labile and more water-soluble "penicillin" also showed the same antibacterial spectrum when tested against *Staphylococcus aureus*, *Streptococcus hemolyticus*, *B. anthracis* and *B. subtilis*, and all were relatively inactive against *Pseudomonas pyocyaneus* and *E. coli*. Because of the great labor otherwise involved these comparisons were not done with sufficient accuracy to show whether differences smaller than 25% exist.

It is then clear that flavicin and penicillin obtained and purified as described above are very closely related, possibly identical, substances. Comparison of pure specimens by more accurate methods is required to demonstrate whether they are identical.

In their recent paper (3) McKee, Rake and Houck describe the production, by various strains of *Aspergillus flavus* grown in submerged cultures, of two antibiotic substances, the aspergillie acid of White (21) (3) and flavicidin, a penicillin-like substance. In these cultures the one or the other type of antibiotic activity predominated, according to the nature of the medium.

Although the flavicidin described was not pure (maximum activity mentioned, 214 Oxford U./mg.) its chemical and antibacterial properties and low toxicity indicate that it is very similar to, or identical with, "flavicin" and "penicillin". The differentiation or identification of the two or more kinds of "flavicin," the two or more kinds of "penicillin," and "flavicidin" (more than one kind?) will require study of more highly purified specimens.

SUMMARY

The application of systematic multiple fractional extraction methods and of chromatographic adsorption procedures to the further purification of flavicin is described. Flavicin having activity of 12,000 *Staphylococcus* Dilution Units per mg. (800 Oxford Units/mg.) has been obtained by both methods. This

material is probably not yet pure. The toxicity in mice of specimens of flavicin having 200-300 Oxford U./mg. is so low that 1000 mg./kg. intraperitoneally produce almost no symptoms. Some pharmacological effects of flavicin are described, including beneficial results in two human patients suffering from *Staphylococcus septicemia*.

Flavicin and penicillin, both purified by the same method to an activity of 500 Oxford Units per mg., are very similar in their chemical and antibiotic properties. Further studies of more nearly pure specimens will be required to demonstrate whether they are identical or not.

We are greatly indebted to Miss Rebecca Taylor and to Miss Charlotte Ward for both chemical and bacteriological assistance.

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THE BIOLOGICAL ASSAY OF EPINEPHRINE

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The purpose of this paper is to describe a procedure for estimating the potency of epinephrine solutions. Certain statistics, calculated from a series of assays performed in the manner to be described, are presented and discussed with relation to the maintenance of a "state of statistical control" in the assay technic.

Various procedures designed to facilitate the calculation of potency of drugs assayed biologically with a graded dose response have appeared frequently in the recent literature (1-5). These procedures are designed to provide for an estimate of the most probable potency and for the determination of the experimental error involved. The assay design to be described below is based upon the precepts of Bliss and Marks (6, 7) and follows a simplified method of calculation devised by Bliss (8).

One of the restrictions involved in the application of an assay design of this type is that it will apply only to graded dose assays where the response has a linear relationship to some function of the dose. Hjort, de Beer and Randall (9) reported a linear relationship between the response of the dog's blood pressure and the logarithm of the dose of epinephrine when the doses employed were sufficient to elicit responses greater than 30 mm. Hg. In the present investigation, the dosages employed were such that the responses were within the range of 30 to 80 mm. Hg, and in many assays the doses were restricted to produce pressure rises of between 30 and 60 mm. Hg, as is specified by the U.S.P. XII (10).

METHOD. The assay procedure as evolved is as follows. A dog of medium size is anesthetized, arranged for recording carotid blood pressure, and the vagal receptor mechanism paralyzed according to the methods described in the U.S.P. XII (10). Several trial doses of diluted reference standard epinephrine are administered at regular intervals of time to determine the doses necessary to produce consistent rises in pressure of between 30 and 80 mm. Hg. From these observations, a dose of diluted standard which will elicit a rise in blood pressure of between 30 and 45 mm. Hg is selected and designated as S_1 , and twice this dose is designated as S_2 . The concentration of unknown is adjusted to be equivalent to that of the standard as nearly as possible. Two doses of unknown dilution equal in volume to those of the standard are selected and designated as U_1 and U_2 respectively. The ratio $S_1:S_2$ and $U_1:U_2$ does not necessarily have to be 1:2. Experience indicates that responses between 30 and 80 mm. Hg are most frequently obtained with this ratio. Regardless of what dosage ratio is selected, however, it must be the same for both standard and unknown, i.e., $S_1:S_2 = U_1:U_2$.

The selected doses of standard and unknown are administered as sets, each set containing all four doses. The four doses within each set are assigned in random order so that all four doses have an equal chance of being assigned to any position within the set. As many sets are employed as is necessary for the required precision. Usually four sets are sufficient to give satisfactory results, although more may be given. The doses are administered at constant intervals of not less than five minutes or at longer intervals depending upon the time required for the blood pressure to return to its former level.

Needless to say, the technic employed to measure the responses must be consistent throughout the assay procedure. The practice followed in these experiments (using an ink writing stylus) was to measure the height of each rise in blood pressure on the kymographic tracing with a millimeter ruler. The difference between the mean systolic pressure immediately preceding a given dose and the maximum rise produced by the dose were the extremes measured. Using a U-type mercury manometer this difference, when multiplied by 2, gives the actual rise in pressure in mm. Hg.

RESULTS. Table 1 includes the protocol of a typical assay and table 2, the calculation of the results. In this assay, the unknown was a dilution of the

TABLE 1
Protocol of assay

2/15/45. Dog no. 443. 7.85 kg. 175 mg./kg. sodium phenobarbital I.P. Reference standard epinephrine 1/100,000. Unknown estimated 1/100,000.

SET NO.	INJECTION NO.	PREPARATION AND DOSE	DOSE	RISE IN PRESSURE
			cc.	mm.
1	1	U_1	0.2	47
	2	S_2	0.4	64
	3	S_1	0.2	47
	4	U_2	0.4	66
2	5	U_2	0.4	72
	6	U_1	0.2	48
	7	S_2	0.4	71
	8	S_1	0.2	47
3	9	U_2	0.4	70
	10	U_1	0.2	53
	11	S_1	0.2	48
	12	S_2	0.4	72
4	13	U_2	0.4	76
	14	S_2	0.4	71
	15	S_1	0.2	54
	16	U_1	0.2	51

standard, the true potency of which was 103.0%. The calculated potency of $103.81\% \pm 3.95\%$ indicates that the accuracy of the method is well within the estimate of its experimental error.

As is customary in testing any new assay procedure, various solutions of known potency were assayed to determine the accuracy of the method. In each case, these known solutions were prepared by dilution of the U.S.P. XII reference standard epinephrine. The true potency was not made known to the analyst until the assay results were calculated. The data from these experiments are tabulated in table 3.

In assay number 9 and number 10 (table 3) the calculated t -value exceeded the tabular value for t indicating that the slopes for standard and unknown were not

parallel. Since the concentrations of standard and unknown dilutions were considerably different in these two assays, one might expect this to occur, and the dilutions should be adjusted to be more nearly equivalent. If, however, a revision of dilutions or of the dosage range fails to produce parallel dosage response curves, as measured by the t -test, one should suspect qualitative differences between the standard and unknown.

TABLE 2
Analysis of data in table 1

SET NO	RISE IN PRESSURE FOR				$D_1 = U_1 - S_1$	$D_1 = U_1 - S_1$	$D_1 = U_1 - S_1$	$D_1 = U_1 - S_1$	$y_1 = D_1 + D_1$	$y_1 = D_1 + D_1$	$y_1 = D_1 + D_1$
	S_1	S_2	U_1	U_2							
1	47	64	47	68	2	0	19	17	2	36	2
2	47	71	48	72	1	1	24	24	2	48	0
3	48	72	53	70	-2	5	17	24	3	41	-7
4	54	71	51	76	5	-3	25	17	2	42	8
Total								$9 = T_1$	$167 = T_2$	$3 = T_3$

N = Number of sets = 4.

I = log ratio of $U_1 \cdot U_2$ and $S_1 \cdot S_2 = 0.201$

$S(y^2)$ = sums of squares of all individual y_1 's, y_2 's and y_3 's

$M = \frac{T_1 I}{T_2} = \log \text{potency}$

Potency = 100 (antilog M)

$b = \frac{T_2}{2IN}$

$s = \sqrt{\frac{S(y^2) - (T_1^2 + T_2^2 + T_3^2)/N}{12(N-1)}}$

$\lambda = \frac{s}{b}$

$S_m = \lambda \sqrt{\frac{1}{N} \left\{ 1 + \frac{T_1^2}{T_2^2} \right\}}$

se = Standard error of relative potency in per cent

$2.303 S_m (\text{antilog } M) (100) = (2 \ 303) (0 \ 01651) (1.0381) (100) = 3.95\%$

Test for parallelism

$t = \frac{T_3}{2s\sqrt{N}}$

$\frac{3}{(2)(2.2868)\sqrt{4}} = 0.3279$

The calculated t should not exceed the tabulated t (see reference 11) which is read with $3(N-1)$ degrees of freedom at $P = 0.05$.

The standardization of the assay technic depends upon whether or not the variability in slope (b), standard deviation (s), and the ratio λ , $\left(\frac{s}{b}\right)$ fall within the limits of random sampling. From twenty-seven assays performed to date in this laboratory the average values for b , s , and λ were as follows: $b = 73.02$ with a standard deviation of 18.75; $s = 2.44$ with a standard deviation of 0.831; $\lambda = 0.0338$ with a standard deviation of 0.0125. These values are probably representative of the variation to be expected in this laboratory but they do not

necessarily indicate the variability in these statistics which may be encountered in other laboratories.

The accumulation of this type of data brings definite advantages to the analyst, for when they are combined with the data from subsequent assays, various terms may fall within the limits of random sampling and the method may be said to be in a "state of statistical control". Control charts (fig. 1) can then be constructed by means of which the agreement of the individual assay is checked with past experience.

In fig. 1, charts are presented to show the variability in b , s and λ . The individual values of b , s and λ have been plotted in the order in which the assays were completed. The control limits which are assigned to each series of statistics were derived by multiplying the standard deviation of each series by 2.0. If

TABLE 3
Results of assays of known solutions

ASSAY NUMBER	TRUE POTENCY	POTENCY FOUND	ACTUAL ERROR AS % OF TRUE POTENCY
	%	%	
1	115	112.8 ± 5.35	1.92
2	122	120.6 ± 5.14	1.15
3	89	88.5 ± 5.32	0.57
4	103	103.81 ± 3.95	0.78
5	119	120.24 ± 5.93	1.04
6	94	94.56 ± 3.73	0.59
7	100	101.4 ± 4.77	1.40
8	77	75.06 ± 4.63	2.52
9	151	153.09 ± 2.91	1.38
10	52	52.05 ± 1.74	0.09
11	83	79.45 ± 2.64	4.28
Mean	..		1.43

these series are representative of the population from which they are drawn, the values of b , s and λ should not exceed by chance their respective control limits more often than once in twenty-two assays (12).

In assay number 14 (fig. 1) the value of λ definitely exceeds its upper control limit. Upon close inspection it is noted that the apparent cause of this deviation from normal is due to a rather large value for s and a rather small value for b in the same assay. This would indicate that the sensitivity of the animal preparation was considerably less than that most frequently observed and that the variation in the individual responses was greater than the average observed in most assays. An inspection of the protocol of the assay revealed that this was the true situation. In this assay the potency had been calculated to be 92.8% $\pm 6.86\%$. This represented a border-line case since the potency would be slightly below the 95% limit allowed by the U.S.P. XII. Because of the large error involved and the failure of λ to fall within its control limits, the assay was

repeated on another animal (Assay 19, fig. 1) whereupon, a potency figure of $91.4\% \pm 2.61\%$ was obtained, and the statistics b , s and λ all fell within their control limits. The preparation was then fortified and upon assay of the adjusted solution a potency figure of $98.1\% \pm 3.82\%$ was obtained (Assay 23, fig. 1).

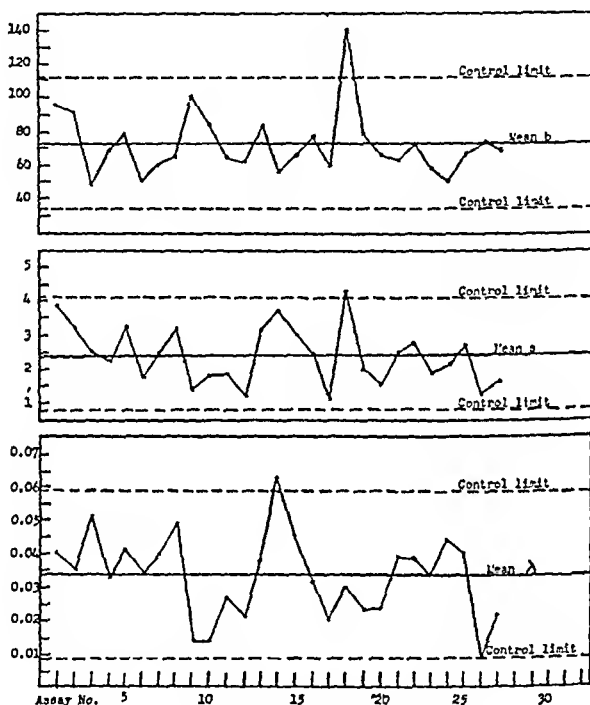


FIG. 1. CONTROL CHARTS FOR b , s AND λ

In assay number 18 (fig. 1) it will be noted that both b and s exceeded their assigned control limits but that λ did not deviate appreciably from the average value for λ . Since both b and s deviate in the same direction, and $\lambda = \frac{s}{b}$, this ratio remains relatively stable when this occurs. It is obvious, therefore, that the maximum control of the assay procedure is maintained only when all three statistics fall within the error of random sampling.

When more data have been accumulated, these control charts will provide additional advantages, for the calculation of the potency and error may become further simplified.

DISCUSSION. Two possible sources of variation are ignored in performing the assay. These are the influence of the preceding dose on a given response, and the influence of the blood pressure immediately preceding a given dose. Analysis of

covariance in several assays shows that these variables are so small that no appreciable increase in precision is obtained from the added calculations. Serious changes in blood pressure throughout the assay period are rarely encountered, and those minor fluctuations which may occur between sets have been isolated from the estimate of potency and error in the assay design.

The use of control charts as described herein permits a more accurate evaluation of the individual assay and, therefore, the calculations of more precise overall results. The advantages that such a tool brings to the analyst are obvious.

In an assay of this type where the error is small, one may wish to calculate only the potency. This can be done rapidly using the formula proposed by Sherwood, Falco and de Beer (4) for the estimation of penicillin potency, or both the potency and error may be calculated with a minimum of effort by employing a graphic method similar to that devised by Knudsen (13) for penicillin assays.

The method is accurate, simple and rapid. The data set forth in table 3 indicate that potency can be determined with an average deviation from true potency of only 1.43 per cent. Anyone familiar with U.S.P. XII assay technic can perform an assay and the calculations involved require only the mathematical substitution in the appropriate formulae. The average time to perform a single assay, including calculations, is approximately four hours.

SUMMARY AND CONCLUSIONS

1. An application of an experimental design to the biological assay of epinephrine has been described.
2. The mean error of eleven assays of solutions of known potency was 1.43%.
3. Control charts are presented and discussed with relation to the maintenance of a "state of statistical control" in the assay technic.
4. The application of the design provides a simple, accurate, rapid and economical method for determining the potency of epinephrine solutions.

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QUANTITATIVE OBSERVATIONS ON MERCUHYDRIN AND MERCUPURIN¹

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The present paper deals with the results of a study of a new mercurial diuretic and a comparison of its diuretic potency and local irritant properties with those of one of the official mercurial diuretic agents in common use, namely, mercurphylline or mercupurin. The new compound is a methoxyoximercuripropylsuccinylurea or mercuhydrin. The material is supplied in the form of a solution of the sodium salt in an ampoule, and each cc. is stated to contain 88 mg. of the compound representing 39 mg. of mercury in organic combination together with 48 mg. of theophylline.

A method for the quantitative comparison of diuretic agents in ambulant patients with heart disease and congestive failure was described in previous studies (1, 2). The patient is weighed. He is then given a dose of the diuretic. The effect is observed in terms of a loss of weight 15 hours after the dose. A week elapses during which edema reaccumulates and weight is regained. The doses are alternated. This procedure may then be repeated as often as is necessary in order to secure a reliable average response to a given dose as a basis of comparison with another compound tested in a similar manner in the same patient. This method has the advantage of testing the various doses in the same patient under substantially similar states of edema, which is not possible when diuretic agents are tested in bed patients.

Our experience with mercuhydrin covers a period of approximately 8 months at the time of this report. It was used in 92 patients and in 43 of these, quantitative comparisons were made with mercupurin. The extent of the present total experience with mercuhydrin in our clinics is shown in table 1. There were 1216 doses of mercuhydrin, by intramuscular and intravenous injection, and during that period 513 doses of mercupurin. The patients were selected from a total case load of 1500 ambulant patients in attendance at the cardiac clinics. All had organic heart disease with advanced congestive failure. All the common varieties of heart diseases were represented, namely, arteriosclerotic, hypertensive, rheumatic, luetic, and hyperthyroid. There were approximately equal numbers of both sexes in ages from 24 to 78 years.

RESULTS. The diuresis after mercuhydrin was compared with that after mercupurin in 43 patients and at three dose levels, 0.5, 1.0 and 2.0 cc. in the case of intravenous injections and 1.0 and 2.0 cc. doses in the case of intramuscu-

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lar injections. The selection of patients for the different doses was based upon previous experience with the particular individual which indicated the dose necessary for a satisfactory diuresis. This experience also showed that the doses did not produce ceiling effects (1, 2). In the previous study (2) it was shown that without the diuretic patients may lose an average of about 0.5 lb. in the 15 hour period between the two weighings. There were no patients who failed to

TABLE 1

Total doses of mercurhydrin and mercupurin in 92 patients

	ROUTE					TOTAL DOSES
	Intravenous			Intramuscular		
	Dose					
	0.5 cc	1.0 cc	2.0 cc	1.0 cc.	2.0 cc.	
Mercuhydrin....	29	163	609	144	271	1216
Mercupurin	35	129	171	94	84	513
Totals	64	292	780	238	355	1729

TABLE 2

Comparison of diuretic effects of mercurhydrin and mercupurin

DOSE	NO OF PATIENTS	NO OF COMPARISONS	AVERAGE WEIGHT LOSS		RATIO OF EFFECT OF MERCUPURIN/MERCURHYDRIN
			Mercupurin	Mercuhydrin	
Intravenous					
cc.			lbs.	lbs.	
0.5	4	14	7.5	6.5	1.16
1 0	21	71	4.0	4.1	0.98
2 0	18	54	4 4	4.2	1.04
All	43	139	4.5	4.4	1.03 \pm 0.06
Intramuscular					
1.0	11	16	3 6	3.3	1.07
2.0	13	19	4.2	4.3	0.98
All	24	35	3.9	3.9	1.01 \pm 0.3

respond to one or another dose, but if one takes all losses of weight above 0.5 lb. as effects of the drug, the results show 4 failures among 139 intravenous injections of mercurhydrin and 3 failures among a similar number of doses of mercupurin, hence failure of response in approximately 2 to 3 per cent of the doses.

The largest diuresis was shown by a loss of 10 lbs. after a dose of mercurhydrin and 12 lbs. after a dose of mercupurin in one case.

The results are summarized in table 2. The average diuresis after 139 intra-

venous injections was similar for the two compounds, namely, a loss of 4.4 lbs. for mercurhydrin and 4.5 lbs. for mercupurin.

The diuretic effects were also similar after the intramuscular doses. The average weight loss in 35 comparisons in 24 patients was 3.9 lbs. for each drug.

Although the average responses to the two drugs were similar, the possibility suggested itself that some patients might respond more favorably to one than to the other drug, and that such preferences might balance each other in the

TABLE 3
Variability in diuretic response

Variability in diuretic response			
COMPARISON NO.	DOSE INTRAVENOUSLY	WEIGHT LOSS	
		Mercurhydrin	Mercupurin
Id Ha			
	cc.	lbs.	lbs.
1	1	9.0	7.0
2	1	5.5	4.8
3	1	2.0	5.0
		16.5	16.8
Es Is			
1	0.5	6.0	10.0
2	0.5	8.5	6.5
3	0.5	5.5	9.8
4	0.5	6.8	10.5
5	0.5	5.5	8.5
6	0.5	8.0	8.5
7	0.5	6.0	6.0
8	0.5	4.5	6.5
		50.8	66.3
Sa We			
1	1	2.5	1.8
2	1	4.0	1.8
3	1	7.3	2.0
4	1	2.5	1.0
5	1	4.0	3.0
6	1	4.8	3.0
7	1	4.0	1.0
8	1	1.5	4.0
		30.6	17.6

average weight loss. The data were therefore examined in more detail for evidence of such preference. Examples of apparent differences are shown in table 3. One patient showed a 15 and 30 per cent greater response with mercurhydrin in the first two comparisons, but in the third comparison the order of preference was reversed, 150 per cent greater response with mercupurin, the average showing no difference between the two drugs. In another patient a series of 8 comparisons showed an average response about 33 per cent in favor of mercupurin, while in a third patient a similar series of 8 comparisons yielded an average

response in the reverse order, about 73 per cent in favor of mercurhydrin. The fact that in one patient 6 of 8 consecutive comparisons favored one drug (mercupurin) and in another patient 7 of 8 favored the second drug (mercuhydrin) reinforced the possibility that differences in the relative efficacy of the two drugs in different patients may exist. It may be noted that by chance alone, it would be expected that one or the other drug would be favored in 7 of 8 comparisons in about 6 per cent of cases, and in 6 of 8 comparisons in about 22 per cent of cases.

A statistical analysis² of all the data was made. The greater diuretic effect was found to occur as often with one drug as with the other, 64 times for mercupurin and 63 for mercuhydrin. The chi square test shows that the high incidence of greater diuretic effect with one or the other drug in some of the patients falls well within the limits of chance.

Renal effects. In 43 patients receiving the mercurials intravenously the urine was examined and the blood NPN determined before the drug was started and at varying periods in the course of treatment. There were in all 94 urine examinations for specific gravity, albumen, casts and cells. There were 89 NPN values. The urines showed no significant changes. The blood NPN during treatment was tested in an average of 13 weeks after the start (3 to 18 weeks). The values in the controls averaged 37.7 mg. per cent (24 to 86) and after an average of about 3 months of treatment 36.2 mg. per cent (22 to 71). There was, therefore, no indication of renal injury after the organic mercurials given intravenously at weekly intervals over periods up to nearly 5 months.

Intensive diuretic therapy. In the routine treatment with organic mercurials many patients are encountered in whom the weekly injection is inadequate. Our past experience with mercupurin and salyrgan-theophylline showed that doses may be given with safety at much shorter intervals over long periods of time. In table 4 our present experience with intensive therapy using mercuhydrin is summarized. There are 13 patients with advanced failure who were maintained in a satisfactory state by intravenous injection of usually 2 cc. 2 times a week, 3 times a week, and at daily intervals over periods of weeks to months.

Systemic reactions. Among the 92 patients involving 1729 injections of the two mercurial diuretic agents there were two patients who developed unfavorable systemic reactions (about 2 per cent). In one there was immediate faintness and giddiness lasting about 30 minutes after 1 and 2 cc. mercupurin intravenously but not intramuscularly. He received mercuhydrin by both routes without reaction. There was the reverse in another case; this patient developed a delayed reaction of chills and fever about 2 hours after the mercuhydrin given intravenously or intramuscularly, although mercupurin was given by both routes without reaction. It has been shown previously (3) that a patient hypersensitive to one mercurial diuretic may take another with impunity.

Local effects of intramuscular injections. It is well known that the organic mercurial diuretics are irritant. They cause a local reaction by intramuscular

² The authors are indebted to Dr. Chester I. Bliss for the statistical analysis.

injection, burning, pain and muscular cramps appearing in from 1 to 3 minutes and lasting from about 5 minutes to about 4 days. A tender nodule may develop in the muscle which persists for months. Observations on the relative intensity of these reactions with the two compounds were made in the course of the present study. The 1 cc. doses were given into the biceps and the 2 cc. doses into the gluteal muscles. Each patients received an injection at weekly intervals for 4 doses. The sites and the drugs were alternated, so that each site received an injection of the two drugs. The reactions were ranked as to severity and duration. There were 65 comparisons (130 intramuscular injections) in 39 patients.

TABLE 4
Intensive intravenous therapy with mercurhydrin

NAME	DIAGNOSIS*	DOSE	TOTAL NO. INJECTIONS	FREQUENCY OF INJECTIONS	DURATION OF TREATMENT
El Pr	AS, Hyper, EH, NSR	2	12	daily	12 days
		2	86	3 weekly	28 weeks
Sa Me	AS, EH, NSR	2	48	2 weekly	24 weeks
Ka Sa	AS, Hyper, EH, BBB	2	46	3 weekly	15 weeks
Wi Ko	Unk, EH, Ao St, AI, MI, NSR	2	9	daily	9 days
Ja Da	AS, Cor Thromb, NSR	1, 2	9	daily	9 days
		1, 2	15	3 weekly	5 weeks
He De	RF, EH, MI, MS, AF	2	40	2 weekly	20 weeks
Mo Lu	Lues, EH, NSR	2	26	2 weekly	13 weeks
Le Jo	Lues, EH, An Ao, NSR	2	26	2 weekly	13 weeks
Be Me	Lues, Hyper, EH, AI, An Ao, NSR	2	26	2 weekly	13 weeks
Gu Na	RF, EH, MI, MS, AI, AF	2	26	2 weekly	13 weeks
Am Sh	AS, Hyper, EH, Cor Scl, NSR	2	26	2 weekly	13 weeks
To Pe	Lues, Hyper, EH AI, AF	2	26	2 weekly	13 weeks
Ab Ma	AS, EH, Cor Thromb, NSR	1	26	2 weekly	13 weeks

* According to "Nomenclature and Criteria for Diagnosis of Diseases of the Heart," of the New York Heart Association (1942): AF (Auricular Fibrillation); AI (Aortic Insufficiency); An Ao (Aneurysm Aorta); Ao St (Aortic Stenosis); AS (Arteriosclerosis); BBB (Bundle Branch Block); Cor Scl (Coronary Sclerosis); Cor Thromb (Coronary Thrombosis); EH (Enlarged Heart); Hyper (Hypertension); MI (Mitral Insufficiency); MS (Mitral Stenosis); NSR (Normal Sinus Rhythm); RF (Rheumatic Fever); Unk (Unknown).

The results strongly favored mercurhydrin. In 3 comparisons (about 5 per cent) the patients interpreted mercupurin as less painful, in 17 comparisons (about 26 per cent) there was no difference, in 39 comparisons (60 per cent) mercurhydrin was interpreted by the patient as causing less discomfort.

Since subjective symptoms and judgments were involved in the foregoing and the preparation injected was known to the observer at the time, the study of the local effects was repeated by the "blind-test". In these experiments the materials were provided in the syringe labeled by letter. The identity of the preparation remained unknown until the data were assembled in the form presented in table 5. There were 20 patients, 35 doses of mercupurin, and 36 of mercurhydrin.

The severity of the reaction was scored from 0 to 4 plus. The 0 indicates no discomfort, the 1 plus barely perceptible discomfort, 2 plus moderate pain, 3 plus marked pain, and 4 plus very severe pain so that the patient protested against repetition of the dose. The "blind-test" study confirms the results of the previous 65 comparisons. In the case of mercurhydrin 55 per cent of the injections produced no discomforts as against 20 per cent for the mercupurin. As to the severity of the local discomfort in terms of the units of measure used in this study, mercupurin scored 3 times as much as mercurhydrin.

TABLE 5

"Blind-test" comparison of local irritant action of mercurhydrin and mercupurin by intramuscular injection

NAME	DOSE	A		B		C		D	
		Pain	Site	Pain	Site	Pain	Site	Pain	Site
He Al	1			1+	LB	2+	RB	0	LB
Na Co	1	1+	RB	0	LB	3+	RB	1+	LB
Ch Co	2	1+	RG	0	LG	0	RG	0	LG
He De	2	4+	RG	1+	LG	4+	RG	4+	LG
Co Fa	1	4+	RB	2+	LB	2+	RB	1+	LB
Ma Ha	2	2+	RG	1+	LG	3+	RG		
Le Jo	2	4+	RG	1+	LG	3+	RG	2+	LG
Fl Le	1	2+	RB			1+	LB	0	RB
Pe Kl	1			0	LB	1+	RB	0	LB
Ar Lo	1	1+	RB	1+	LB	1+	RB	0	LB
Be Mc	2	0	RG	0	LG	0	RG	0	LG
Th Mc	1					0	RB	0	LB
Bl Mi	2	4+	RG	1+	LG			1+	RG
To Pe	2	4+	RG	0	LG	1+	RG	0	LG
Al Qu	1	1+	RB	0	LB	1+	RB	0	LB
Na Sa	2	1+	RG	0	LG	0	RG	0	LG
Ma Sp	2	0	RG	2+	LG	3+	RG	3+	LG
An Wa	1			1+	LB	2+	RB	1+	LB
Be Wi	2	1+	RG	0	LG	0	RG		
Li Wy	1	3+	RB	0	LB	1+	RB	0	LB
Average		2 1+		0 6+		1.5+		0.7+	

A and C are mercupurin, B and D are mercurhydrin, R (right); L (left) B (biceps); G (gluteal)

SUMMARY AND CONCLUSIONS

1. A method is described for the quantitative comparison of diuretic agents in ambulant patients with congestive heart failure.

2. Experience is described in the use of a new mercurial diuretic, mercurhydrin, during a period of 8 months in 92 patients who received a total of 1216 doses of 0.5, 1 and 2 cc by intravenous and intramuscular injection.

3. In the dosage range of 1 and 2 cc, the diuretic responses to mercurhydrin and mercupurin by intramuscular and intravenous administration are indistinguishable.

4. Sources of error in the quantitative comparison of diuretic agents are indicated. It is demonstrated how pure chance may prove grossly misleading in individual cases.

5. Neither of the two agents produces renal injury as shown by urine examinations and blood NPN values after weekly injections for periods of 3 months.

6. A comparison with a "blind-test" method shows that mercuhydrin is less irritant than mercupurin by intramuscular injection.

7. Hypersensitivity to either of these agents may not necessarily extend to the other.

The authors are indebted to Miss Elizabeth Blake, Miss Sarah Oxfield and Mrs. Samuel Chotzinoff for generous assistance in the course of the study.

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ON THE ACTION OF MARFANIL AND OTHER ANTICLOSTRIDIAL AGENTS ON ANAEROBIC BLOOD AGAR PLATES¹

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Since the first demonstration by Chain and coworkers of the effectiveness of penicillin in experimental *Cl. septicum* infections (1), the study of anticlostridial agents has been further stimulated by Klarer's (2) and Domagk's (3) reports of a specific activity against pathogenic clostridia shown by p-(amino-methyl)-benzene sulfonamide hydrochloride² ("Marfanil"). It has been found that this drug is not counteracted by p-aminobenzoic acid (4) and that its useful effectiveness is limited to local application (5) (6).

While sulfonamides of the sulfanilamide type have been extensively studied with regard to their absorption and excretion in man and in experimental animals, no similar information on Marfanil is available as yet. This is mainly due to the fact that this drug cannot be determined chemically by means of Marshall's colorimetric method (7). An attempt was therefore made to work out a biological method for determining Marfanil in body fluids and tissue extracts.

Under (A) the experimental basis of this test is presented and its application illustrated by recording observations on the absorption and excretion of Marfanil in mice. Under (B) observations are described which demonstrate the possibility of evaluating the anticlostridial activity of various other therapeutic agents by means of anaerobic blood agar plates.

A. BIOLOGICAL METHODS FOR DETERMINING MARFANIL IN MUSCLE AND BODY FLUIDS. *Principle.* Blood agar plates, seeded with certain pathogenic clostridia show marked hemolysis on anaerobic incubation. If small filter paper discs soaked with aqueous solutions of Marfanil are placed on such plates before incubation, the Marfanil, diffusing into the medium, prevents the growth of clostridia, and thereby produces circular areas in which the hemolysis is inhibited (see fig. 1). The size of these inhibition zones is related to the concentration of the Marfanil solutions placed on the discs. The unknown concentration of Marfanil in blood, urine or tissue extracts can therefore be determined by placing filter paper discs soaked with these fluids side by side with discs containing known concentrations of the drug.

The principle applied in this method is similar to that used in the determination of penicillin by means of the filter paper disc modification (8, 9) of Heatley's original method (10). But in the case of Marfanil we use an anaerobe as test organism.

¹ This investigation was assisted by a grant in aid from the National Research Council in Ottawa.

² This drug was synthesized in our laboratory in collaboration with John R. Polley, M.A.

Technique. Best results were obtained by using paper discs of 8 mm. diameter, cut out of Whatman filter paper no. 2 by means of a cork borer. The discs are sterilized in an autoclave.

As judged by the size of the inhibition zones, *Cl. histolyticum*, used as a test organism (table 1, fig. 2) proved to be a more sensitive indicator for the presence of Marfanil than *Cl. welchii*, and has been chosen as the test organism.

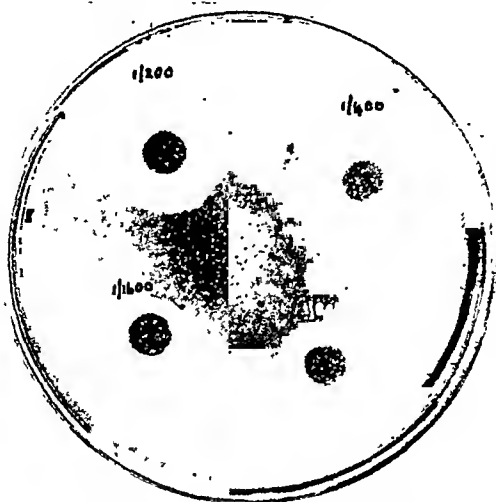


FIG. 1. Anaerobic blood agar plate, prepared with beef infusion plus 1% Parke Davis peptone, seeded with *Cl. welchii*, showing zones of inhibition of hemolysis, as produced by Marfanil. Filter paper discs contain Marfanil in dilutions 1:200-1:1600. (Plate illuminated by direct and transmitted light combined).

The medium used for preparing the deep agar plates is constituted as follows:

- 13 cc. tryptic beef digest, containing 2% agar
- 1 cc. defibrinated human (or sheep's) blood.

This mixture, contained in a test tube, is seeded by adding, immediately before pouring the plate, 0.1 cc. of a spore suspension of *Cl. histolyticum* in 0.8% saline. The latter was prepared as follows:

Cl. histolyticum (strain 8213) was grown anaerobically for 4 days at 37°C. on Dorset's sugar free egg medium. The growth, after being washed and resuspended in saline, was heated at 75°C. for 1 hour in order to kill any vegetative forms. Of this suspension, 0.1 cc. of a dilution 1:10 was added to 14 cc. blood agar, giving approximately 40,000 colonies per plate.

In order to preclude the possible interference with the Marfanil test of sulfonamides of the sulfanilamide type, if present, a small amount of p-aminobenzoic acid (0.001%) may be added to the medium.

For setting the paper discs on the blood agar plates, it is important that the discs, containing the solutions³ to be tested, be placed side by side with the discs holding the Marfanil standard dilutions. The plates are then incubated anaerobically (hydrogen atmosphere) at 37°C. for 20 to 24 hours.

³ In the early experiments the volume of the solutions to be tested (0.02 cc.) was accurately measured. Later experiments showed that soaking the discs with the solutions and removing excess liquid by touching the wall of the test tube with the filter disc gave equally satisfactory results.

TABLE 1

Demonstration of antihemolytic effect of Marfanil on anaerobic blood agar plates, seeded with *Cl. welchii* or *Cl. histolyticum*

TEST ORGANISM	MARFANIL		INHIBITION ZONES	
	Dilution	Mg. %	Diameter mm.	Degree of inhibition
<i>Cl. welchii</i> *	1:200	500	25	Complete
	1:400	250	20	Complete
	1:800	125	16	Complete
	1:1600	62.5	13	Complete
<i>Cl. histolyticum</i> †	1:800	125	26	Nearly complete
	1:1000	62.5	21	Nearly complete
	1:3200	31	18	Nearly complete
	1:6400	15.5	15	Partial
	1:12,800	7.8	12	Partial

* *Cl. welchii* (Type A) was grown for 4 hrs. at 37°C. in modified Robertson's medium. Of this culture 0.1 cc., diluted to 10⁻³ with saline, was added to 14 cc. of blood agar (containing beef infusion + 1% Parke-Davis peptone). A dilution plate count showed the presence of approximately 40,000 clostridia per plate.

† For preparation of the *Cl. histolyticum* plates, see under technique.

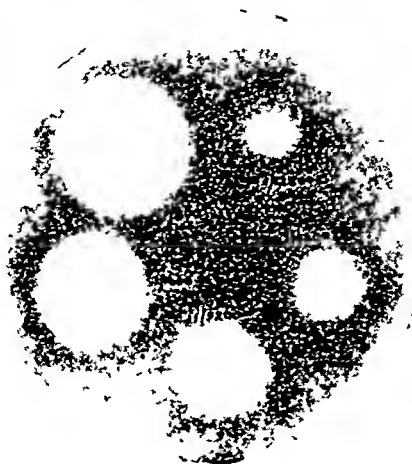


FIG. 2 Anaerobic blood agar plate, prepared with tryptic beef digest, seeded with *Cl. histolyticum* showing zones of inhibition of hemolysis as produced by Marfanil. Filter paper discs contain Marfanil in dilutions 1:800-1:12,800. (Plate illuminated by transmitted light only.)

In using the above technique, duplicate determinations on any Marfanil solution, containing from 10–100 mg. % of the drug, show maximum variations of 3 mm. in the diameter of the inhibition zones. As in the case of penicillin, determinations of Marfanil based on single plate readings, are therefore approximate, yet sufficiently accurate to indicate the trend in absorption and excretion of this drug in the animal body.

Absorption of Marfanil in mice. For studying the absorption of Marfanil after intramuscular administration, groups of white mice (20–24 g.) were injected into the right thigh with 0.1 cc. of an aqueous solution containing 10 mg. Marfanil. At various time intervals after administering the drug, the mice were killed by means of chloroform. The whole thigh muscle was cut out and was

TABLE 2

Absorption and excretion of 10 mg. Marfanil after intramuscular and oral administration to mice

TIME AFTER DRUG ADMINISTRATION (10 MG.)	MARFANIL CONCENTRATION			
	After intramuscular injection			After oral administration
	Right thigh av *	Blood av	Urine av.	Blood av.
	mg	mg %	mg %	mg. %
7 min	5.4	10	187 (3)	0 (2)
30 min	5.1	14	3,000 (3)	5 (2)
1 hr	1.4	10	1,000 (2)	12 (5)
2 hrs	0.4	6	2,000 (3)	7.5 (2)
3½ hrs	0	3.7	2,500 (3)	7.5 (5)
6 hrs	0	0 (3)	375 (2)	10 (2)
16 hrs	0 (2)	0 (3)	0 (2)	0 (2)
24 hrs			0 (2)	0 (2)

All figures, without indices, recorded in columns 2–5 are averages based on results obtained with groups of 4 mice. Where larger or smaller groups were used, the number of mice are recorded in brackets.

* The Marfanil content of the right thigh muscle is recorded as total amount of the drug present in the whole thigh

thoroughly ground up in a mortar with sterile sand after adding enough saline to correspond to three times the weight of the thigh muscle (0.5–0.8 g.). The mixture was then centrifuged and the supernatant solution tested for its Marfanil content, using the above described technique. Immediately before killing the mice, blood and urine samples were taken and their Marfanil content determined. The blood was used undiluted and was drawn directly from the tail onto the filter discs. The urine however was, as a rule, diluted from 10 to 100 times before being placed on the discs, as the Marfanil concentration in urine may reach very high levels. Neither blood nor urine of normal mice proved to have any antibacterial action against *Cl. histolyticum*.

From the results of the Marfanil determination in the thigh, blood and urine, as recorded in table 2, it may be seen that this drug is rapidly absorbed into the

blood stream forming maximum blood levels in less than one hour after injection. After six hours, no detectable amounts of Marfanil are present in the blood, while the urinary excretion is completed in less than 16 hours.

The absorption of Marfanil after oral administration was studied in a similar way and the corresponding blood concentrations are entered in table 2 for comparison. The orally administered drug seems to form somewhat lower blood levels, which however are somewhat more sustained than in the case of intramuscular injection. Nevertheless, like sulfanilamide (11), Marfanil is rapidly absorbed after oral administration.

In view of the low anticlostridial activity of Marfanil after systemic administration (6), it appeared of interest to obtain information regarding the change this drug undergoes in the animal body. Experiments were therefore carried

TABLE 3
Urinary excretion of Marfanil in mice

DRUG ADMINISTRATION	MARFANIL RECOVERED	
	In 24 hours urine	Excretion (average)
10 mg. per os	mg.*	%
	1.3	20.4
	1.6	
	1.2	
	1.0	
	1.7	
	3.0	
10 mg. i.m.	4.5	
	2.2	31.3
	4.4	
	2.8	

* Each figure represents titration for single mouse.

out in which the 24 hour urine of mice was collected after intramuscular or oral administration of 10 mg. Marfanil. The results recorded in table 3 indicate that on the average less than one third of the administered drug is excreted in active form. That this result is not due to acetylation of Marfanil, was demonstrated by the observation that the anticlostridial activity of the urine, as measured on blood agar plates, was not changed by 30 minutes hydrolysis at 100°C. in the presence of 0.5 normal hydrochloric acid. There must therefore exist other forms of degradation processes which account for the rapid inactivation of Marfanil in the animal body. In this connection the finding of Beyer and Govier (12) that Marfanil can be oxydized "in vitro" in the presence of amine-oxydase is of special interest.

B. EVALUATION OF ANTICLOSTRIDIAL ACTIVITY OF THERAPEUTIC AGENTS BY MEANS OF BLOOD AGAR PLATES. Anaerobic blood agar plates as used by us for determining Marfanil in body fluids, were also used for studying the effect of

various other therapeutic agents upon pathogenic clostridia. The observations, reported here, deal exclusively with the strain of *Cl. welchii* used in "in vivo" tests (6).

Filter paper discs (8 mm. diameter) saturated with aqueous solutions of the agents to be tested, were placed on blood agar plates, previously seeded with *Cl. welchii* as described above. The plates were then anaerobically incubated for 18 hours at 37°C. Great differences were noticed between various therapeutic agents with regard to their ability of producing circular areas in which either bacterial growth or hemolysis or both were inhibited (table 4 and fig. 3)

TABLE 4

Effect of various therapeutic agents on *Cl. welchii* grown on anaerobic blood agar* plates

THERAPEUTIC AGENT (0.02 CC.) PLACED ON FILTER PAPER DISCS	DILUTION IN 0.8% SALINE	INHIBITION ZONES (18 HRS. INCUBATION)			"IN VIVO" ACTIVITY AGAINST <i>CL.</i> <i>WELCHII</i> IN MICE†
		Diameter†	Inhibition of		
			Growth	Hemolysis	
		mm			
Marfanil	1:200	26, 23, (25), (23)	Complete	Complete	Marked
	1:400	20	Complete	Complete	
	1:800	13	Complete	Complete	
	1:1600	11	Complete	Complete	
	1:3200	0	None	None	
Sulfathiazole (sod salt)	1:200	0, 0, (0)	None	None	Slight
Sulfadiazine (sod salt)	1:200	0, 0, (0)	None	None	Slight
Penicillin (sod salt)	5 units per cc	19, 20, (21), (19)	Complete	Complete	Marked
Perfringens antitoxin	225 I U per cc	15, (15)	None	Nearly complete	Marked

* Tryptic digest medium containing 2% agar and 8% defibrinated (human) blood.

† These figures include the diameter of the paper discs (8 mm), except where no inhibition zone was formed—Figures in brackets refer to duplicate tests on blood agar plates, containing 0.01% p-aminobenzoic acid

‡ Referring to results (6) obtained in experiments with mice, intramuscularly infected with *Cl. welchii* and treated either locally with sulfonamides or penicillin or systemically with antitoxin

In using tryptic beef digest as a basic medium it first was noticed that Marfanil produced essentially the same inhibition areas (table 4) as those observed when beef infusion was used for preparing the blood agar plates (table 1). In contrast to Marfanil, neither sulfathiazole nor sulfadiazine had any effect on the growth of *Cl. welchii*, numerous colonies developing freely in the vicinity of the paper discs as throughout the plate, producing complete hemolysis. Penicillin,⁴ however, in a concentration of 5 units per cc., produced like Marfanil an area of total growth inhibition, in which hemolysis was completely prevented.

As might be expected, perfringens antitoxin horse serum⁴ (table 4 and fig. 3),

⁴ As prepared by the Connaught Laboratories.

owing to its content of anti-hemolysin, produced a red opaque inhibition area. On microscopic examination, however, this inhibition zone characteristically differed from the inhibition zones formed by penicillin and Marfanil. In the case of the latter, the areas in which hemolysis was prevented, coincided with the areas of growth inhibition, while in the inhibition zone formed by the antitoxin, no growth inhibition took place, the colonies of *Cl. welchii* being present in approximately the same number and size as throughout the plate. The red blood cells appeared intact except in the peripheral area of the inhibition zone, where some of the outer colonies were surrounded by small hemolytic clearances.

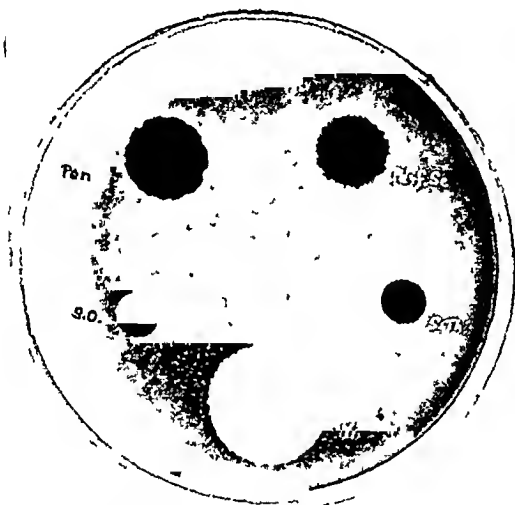


FIG 3 Anaerobic blood agar plate, prepared with tryptic beef infusion and seeded with *Cl. welchii*, showing inhibition zones as produced by Penicillin, 5 units per cc (Pen), sulfadiazine (S D), Marfanil (M) and sulfathiazole (S Th) all at a dilution 1:200 (\approx 5 mg per cc). The Perfringens antitoxin (Perf Se) contained 225 I U per cc.

By means of microscopic examination of the inhibition zones, one can therefore clearly differentiate between the antibacterial factor and the anti-hemolytic factor, only the latter being present in the antitoxic serum, while in the case of Marfanil and penicillin the antibacterial factor fully accounts for the absence of hemolysis, which is the consequence of growth inhibition.

Correlation between "in vitro" and "in vivo" activity. The two following questions arise. The first is whether such plate experiments lend themselves to a quantitative evaluation of the "in vitro" activity of chemotherapeutic agents, and the second whether there is a close correlation between the size and the character of the inhibition zones and the "in vivo" activities shown by the same therapeutic agents when tested in experimental animals or man.

As regards the first question, only in the case of different therapeutic agents

producing inhibition zones of similar character should a quantitative comparison of their relative "in vitro" activities be attempted. Such comparable zones, showing complete inhibition of growth and hemolysis, are formed by penicillin and by Marfanil. From table 4 may be seen that a solution, containing 5 units per cc. of penicillin, forms an inhibition zone of 20 mm. diameter. To produce an inhibition zone of the same size by means of Marfanil a dilution of 1:400 of this drug, corresponding to 2.5 mg. per cc., is required. As judged by the bacteriostatic effect on *Cl. welchii*, grown on blood agar plates, 5 units of penicillin (corresponding to 0.003 mg. of the Na-salt) are therefore equivalent to 2.5 mg. of Marfanil. This indicates that under the conditions of the plate test and on a weight per weight basis, penicillin is approximately eight hundred times as effective as Marfanil.

In a previous study (6) it was shown that in the early local treatment of experimental gas gangrene produced in mice by intramuscular infection with *Cl. welchii*, the Na-salt of penicillin is about 60 times more effective than Marfanil. Both the "in vivo" and the "in vitro" experiments agree, therefore, in that they demonstrate a marked superiority of penicillin over Marfanil. But in the "in vivo" experiments this superiority is less pronounced than in the plate test.

Some divergence between the results of "in vivo" and the "in vitro" tests may readily be expected as under "in vitro" conditions the influence of important factors like phagocytosis or the rate of absorption and excretion of the therapeutic agents is not measured. In contrast to antibacterial "in vitro" tests, carried out in test tubes with fluid media, the above described plate tests do, however, take into account the influence, on antibacterial activity, of the rate of diffusion of the therapeutic agent, a factor which plays an important part during local chemotherapy applied at the site of infection.

The absence of an anticlostridial activity of sulfathiazole and sulfadiazine, when tested on blood agar plates as recorded in figure 3, is likely due to the presence in the medium of a small amount of sulfonamide inhibitor, which was detected by testing the basic medium (tryptic beef digest or beef infusion + 1% Parke-Davis peptone) by means of the method described by Macleod (13). The correctness of this conclusion was confirmed by the observation that these two sulfonamides, when tested on blood agar plates, containing as basic medium liver infusion (prepared according to MacLeod), produced large zones of 30 mm. diameter in which growth and hemolysis were partly inhibited (see fig. 4). In confirmation of a report by the same author, we found beef liver extract to be practically free of sulfonamide inhibitor.

Such an inhibitor-free medium does however represent growth conditions which differ from those prevailing in muscle tissue, where small amounts of inhibitor are likely to be present and may reduce the effectiveness of sulfonamides. Furthermore, the inhibition areas formed by Marfanil on the "blood-agar-liver" medium, seeded with *Cl. welchii*, were regularly found to be smaller than those produced by this same drug on blood agar containing tryptic beef digest (see figs. 3 and 4), while the inhibition zones formed by penicillin were of about the same size on the two media. This observation suggests that liver extract

contains a constituent which interferes with the anticlostridial action of Marfanil.

By working with a basic medium, containing, like muscle tissue, small amounts of sulfonamide inhibitor as in the case of the plate experiments recorded in table 4, one can demonstrate by means of one single blood agar plate (fig. 3), that penicillin and Marfanil have a marked anticlostridial effect under conditions under which neither sulfathiazole nor sulfadiazine show any activity against *Cl. welchii*.

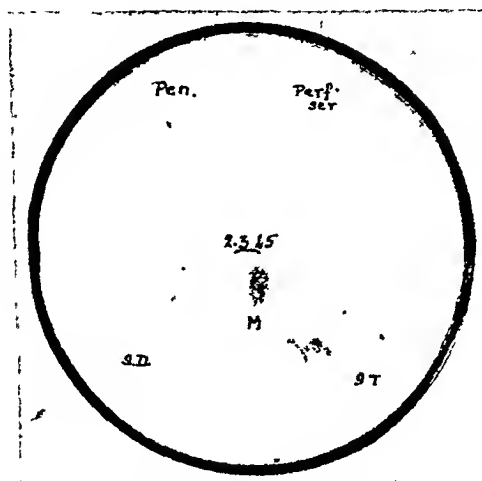


FIG. 4 Anaerobic blood agar plate, seeded with *Cl. welchii* showing similar experiment as in fig. 3, but using liver infusion as basic medium.

A comparison between the "in vitro" and the "in vivo" tests as recorded in table 4, cols. 4, 5 and 6, shows that those therapeutic agents which exhibit an "in vitro" activity prove to have a definite though limited effectiveness in experimental *Cl. welchii* infection in mice. This applies to penicillin, Marfanil and perfringens antitoxin, while sulfathiazole and sulfadiazine, though inactive according to the plate test (fig. 3), still show a slight activity in the animal experiment. But the uncertainty as to the amount of sulfonamide inhibitor present in infected muscle and the finding of Reed and Orr (14) that certain strains of *Cl. welchii* readily produce p-aminobenzoic acid during growth, stress the importance of using for the treatment of gas gangrene such therapeutic agents which are not counteracted by p-aminobenzoic acid.

The study of the anticlostridial activity of therapeutic agents by means of anaerobic plates is being pursued for other pathogenic clostridia.

CONCLUSIONS

1. A biological test for Marfanil, based on its anticlostridial action against *Cl. histolyticum*, was worked out.

2. The study, by means of this test, of the absorption and excretion of Marfanil in mice indicates that this drug is rapidly absorbed after intramuscular or oral administration, but that only a small portion of the Marfanil is excreted in the urine in active form.

3. A study was made, by means of anaerobic blood agar plates, of the anticlostridial and antihemolytic action on *Cl. welchii* of Marfanil, penicillin, sulfathiazole, sulfadiazine and perfringens antitoxin. In applying the paper disc technique as used for testing penicillin on plates, the influence of different media on the anticlostridial activity of the various agents was investigated.

A basic medium like tryptic beef digest, containing small amounts of sulfonamide inhibitor, approaches more closely "in vivo" conditions, prevailing in infected muscle tissue, than does inhibitor free liver infusion. In the former medium, the typical sulfonamides are ineffective while penicillin and Marfanil produce large zones in which growth and hemolysis are inhibited. On a per weight basis penicillin is several hundred times more effective than Marfanil.

On the "blood agar-liver" medium sulfathiazole and sulfadiazine show a marked anticlostridial activity as against a slight effectiveness in the local treatment of the experimental infection in mice.

Penicillin shows an equally marked "in vitro" activity on both media. The liver infusion does however contain a factor interfering with the activity of Marfanil.

Perfringens antitoxin, tested on either medium, has exclusively an antihemolytic but no antibacterial action.

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STUDIES ON ANALGESICS

I. THE TIME-ACTION CURVES OF MORPHINE, CODEINE, DILAUDID AND DEMEROL BY VARIOUS METHODS OF ADMINISTRATION

II. ANALGESIC ACTIVITY OF ACETYSALICYLIC ACID AND AMINOPYRINE

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PART I. 1. TIME-ACTION CURVES OF MORPHINE ASCERTAINED BY VARIOUS METHODS OF ADMINISTRATION. While numerous data have been obtained in several species on the duration as well as the intensity of analgesia produced by morphine, so far, no exact determinations seem to have been made of the relationship between dosage and duration and intensity of action. No doubt, this is due to the difficulties which one encounters when trying to determine analgesia quantitatively and, especially, when making repeated determinations at short intervals. Probably for similar reasons, the comparative analgesic effects produced in the same species by different methods of administration have not been investigated so far.

For data on morphine analgesia in a number of species, we may refer here to the monograph by Eddy (1).

By using the method of pain stimulation by thermal radiation (2), Hardy, Wolff, and Goodell succeeded in determining on humans accurate time-action curves of opiates (3). Later, this method was used on animals by d'Amour and Smith (4), and by Andrews and Workman (5).

In the present report, a new adaptation of the Hardy-Wolff method will be described by presenting time-action curves of morphine obtained on rats to whom the drug was administered by various routes. With this new method which allows accurate measurement of the pain threshold on experimental animals, observations may be repeated at very short intervals.

Material and Methods A shaved area of the back of the animal was exposed to a constant heat (pam) stimulus obtained with the apparatus illustrated in figure 1. Onset, duration, and intensity of analgesia were determined by measuring the time ("reaction time") elapsing between beginning of exposure and response of the animal to the stimulus.

Technique By means of the transformer, the voltage may be adjusted at any desired level. The whole set-up is so arranged that the concentrated light beam is centered exactly on the opening in the lucite screen (Eccentric focusing might fail to give the exact reaction time). An asbestos screen is interposed between lamp and lens and serves as a shutter. The use of dark glasses is advisable. The shaved area is placed against the opening in the lucite screen. After removing the shutter, the reaction of the animal to the heat stimulus is observed. The reaction time is accurately determined. Repeated tests may be made on different skin areas.

It was found in preliminary tests that older rats (300 to 500 grams) showed (irregular) fluctuations of their pain threshold level, while younger rats did not. In consequence, rats of 100 to 200 grams body weight were used in all tests.

Season, room temperature, diet, even incomplete diets producing vitamin A or B₁ deficiencies¹ seemed to exert no influence on the response.

Observations made in only the first set of tests were used for these records. It had been observed before that after an animal had once been treated with opiates, its manner of reaction in future tests might be influenced by the previous treatment.² Tests were made on 2200 albino rats, males and females. Approximately 50,000 single tests were made during three and a half years, and about 3500 individual time-action curves were established.

Morphine hydrochloride, codeine phosphate, and dihydromorphinone hydrochloride (dilaudid), resp., were used. Doses are expressed in terms of their alkaloid bases. For demerol, the hydrochloride of ethyl ester of 1-methyl-4-phenyl-4-carboxylic acid, figures are given in terms of the hydrochloride.

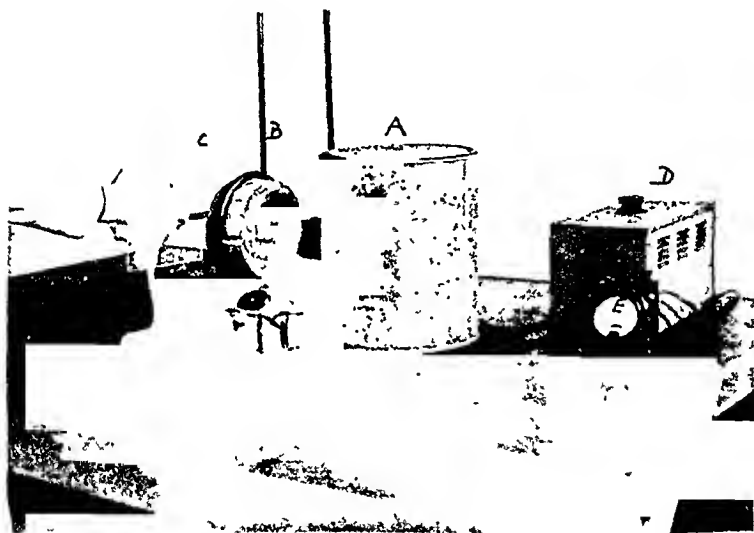


FIG 1. SET-UP OF THE EXPERIMENT

- (A) Radiation source: Mazda lamp, 1000 Watts, covered for the protection of the investigator with a metal box having a window.
- (B) Biconvex lens, 10.2 cm. diameter—focal distance 13.5 cm. Concentrates the light of (A) on
- (C) Lucite screen, with a circular opening of 1.5 cm. diameter in the center
- (D) Transformer, attached to street current
- (E) Voltmeter, inserted between (D) and (A).

EXPERIMENTAL PART. *The Reaction-Pattern of Untreated Animals and Humans under Thermal Radiation.* Normal rats show two distinct sets of reactions: (a) twitching of the skin, this being quite evident over the irradiated zone; (b) retracting the entire body and attempting to escape, or the latter response only. To all appearances, these movements represent a fight-flight type of reaction.

¹ We are indebted to Mr. Dreckler of the Roche Nutrition Laboratory for supplying us with rats kept on an avitaminotic diet.

² This observation is of interest in reference to tolerance formation.

Usually, reactions (a) and (b) occur simultaneously or in close sequence, the twitching reaction then slightly preceding the escape movement by 0.1 to 0.2 seconds. Table 1 records the reaction times of untreated rats as observed by several investigators. The constancy of the reaction time—with the majority of animals reacting after 4 to 5 seconds under our experimental conditions—is very definite.

Hamsters, guinea-pigs, and cats also were found suitable for the heat test, while mice when irradiated on either the dorsal or ventral side showed no regularity in their response.

By experimenting on humans—focusing the light beam on the dorsum of the hand—it was possible to ascertain which voltage would incite the most precise reactions, thereby reducing the range of experimental error to a minimum. With low voltages, from 40 to 50 volts, perception of pain was indistinct and accompanied by heat sensation. In consequence, the range of fluctuation was wide (about 50%) when repeated determinations were made on the same subject. With higher voltages (100 volts or more), perception of pain was very

TABLE 1

Reaction time of untreated rats determined by the method of thermal radiation
Voltage used · 85

Criterion of response · twitching of the skin

NO. OF RATS	NO. OF DETERMINATIONS	REACTION TIME IN SECONDS					
		2	2.5-3.5	4-5	5.5-6	6.5-7	7-8
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
2134	4050	0 22	25 75	63 13	9 92	0.91	0.05

precise. However, the percentage of error (0.5 seconds in 2.9) still remained high in view of the shorter exposure.

The use of 85 volts with an average reaction time of 4 seconds produced the smallest range of experimental error (10%). With such a current, pain occurred suddenly in the form of a prickling sensation not preceded by any heat sensation. Lengthening the exposure time by 25% produced a "burning" sensation. By doubling the exposure time from 4 to 8 seconds, pain sensation became pronounced. After exposure for 14 seconds, an intensely painful burning sensation was experienced, and blisters resulted.

Influence of Morphine on the Reaction Pattern of Rats. Rats were given morphine by subcutaneous, intraperitoneal, intravenous, or oral administration and submitted to the heat test. Their individual reaction patterns were studied.

(a) *Subcutaneous injection.* The degree of analgesia was measured at 15, 30, 45, 60, 90, 120, and 180 minutes after injection. In cases in which analgesia extended over more than three hours, tests were continued at 30-minute intervals until the reactivity of the animal dropped back to the original threshold level.

It was necessary to ascertain the shortest reaction span at the end of which complete analgesia was established. This had to be done for the purpose of

making the data strictly comparable. If no response occurred within 16 seconds' exposure time, none was to be expected later, since undoubtedly analgesia was then complete. Animals not reacting after 16 seconds could be severely burned without giving any reaction. In cases where there was a response before 16 seconds, the degree of responsiveness between 10 and 15 seconds varied considerably, as it did in all cases below 10 seconds.

It was concluded that *complete analgesia* has been established when there is no reaction after 15 seconds of exposure. In calculating all the averages appearing in this report, the maximum reaction time under treatment was considered to be 15 seconds. In more recent experiments, such a waiting period was arbitrarily set as the limit of observation. The subjective experience of pain reported for humans justifies such a procedure.

Table 2 gives the average reaction times (twitching) observed in rats after the subcutaneous administration of from 3 to 20 mgm./kgm. morphine. The

TABLE 2
Average reaction time of rats treated subcutaneously with morphine

DOSE	NO. RATS TREATED	REACTION TIME (IN SECONDS) AFTER HOURS										
		Before	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$	1	1 $\frac{1}{2}$	2	2 $\frac{1}{2}$	3	4	5
mgm./kgm.												
3.0	10	3.8	3.9	3.7	3.9	4.1	3.9	3.8	3.9			
4.0	37	5.08	6.04	6.57	6.88	5.93	4.92	5.83	3.55	4.67		5.75
5.0-6.0	100	4.59	6.61	8.05	7.85	7.12	6.19	6.20	6.43	5.61	4.58	5.0*
7.5	115	4.79	7.15	9.03	10.23	9.46	8.71	7.23	7.64	7.61	4.07	4.19†
10.0-12.0	95	4.24	6.55	10.24	9.48	10.89	9.84	8.8	6.64	5.8	4.51	5.12
15.0	25	4.26	5.26	9.22	12.5	13.70	10.05	12.0	5.10	5.5	4.77	4.64
20.0	15	4.46	7.0	13.8	13.6	15.0	13.5	13.6	10.8	9.5	8.7	4.8

* Average of 37 rats.

† Average of 64 rats.

minimum analgesic dose (M.A.D.) is 4 mgm./kgm., giving an average (peak) reaction time of 6.88 seconds. It appears that this time-span increases in proportion to the doses administered. Doses of 4 mgm./kgm. produce an average increase over the normal threshold of 35%; doses of 5 to 6 mgm./kgm., an increase of 71%; doses larger than 7.5 mgm./kgm., an increase of over 100%. The administration of 20 mgm./kgm. brings about complete abolition of all reactivity (an average increase over the normal threshold of more than 200%).³

Table 3 gives the numerical distribution of morphine-treated rats according to the maximum analgesic effect reached. A definite quantitative relationship exists between dosage size and number of rats showing moderate, high, intense and complete analgesia.

We define as the *average analgesic dose* (A.A.D.) of a drug the dose which in our experiments induces measurable analgesia (reaction time within 6 and

³ We define as threshold of reaction the length of heat exposure required to bring about response.

14 seconds) in approximately 40 to 60% of the group, while the remaining animals are uniformly distributed between "zero and complete analgesia". This average dose appeared to be the most characteristic one. It was possible to determine it on a very small number of animals. With reference to the twitching reaction, the minimal analgesic dose is 4 mgm./kgm.; the average, 6 mgm./kgm.; the "complete analgesic dose", which gives complete analgesia in the majority of animals, 15 mgm./kgm.

If the flight ("voluntary") reaction rather than the twitching ("reflex") reaction is taken as the standard gauge, a higher figure for threshold elevation will be obtained.⁴ Treatment with opiates will lengthen the normal interval between "reflex" and "voluntary" reactions. In untreated animals, the two figures fall almost within the range of experimental error. Thus, when the flight reaction was taken as criterion, the doses with which "analgesia" was established were smaller than when waiting for the twitching reaction.

TABLE 3

Distribution of rats injected with 3-20 mgm./kgm. morphine (s.c.) according to the intensity of analgesic reaction

DOSE MORPHINE (BASE)	NO RATS	NO ANALGESIA	20-30% INCREASE OF THRESHOLD MAX REACTION TIME UNDER 6 SEC (±)	REACTION TIME 6-8 SEC "MODERATE"	REACTION TIME 9-12 SEC "HIGH"	REACTION TIME 13-15 SEC "INTENSE"	REACTION TIME OVER 15 SEC "COMPLETE"
mgm /kgm s.c.		per cent	per cent	per cent	per cent	per cent	per cent
3.0	10	100					
4.0	37	22	8.5	33.3	30.5	2.7	2.7
5.0-6.0	100	10.0	9	39	12	8	22
7.5	115	5.3		19.6	18.8	9.8	46.5
10-12.0	95	4.2	3.2	21.7	6.4	6.4	58
15.0	25			4	8		88
20.0	15						100

Onset and duration of analgesia. Following subcutaneous injection of morphine, analgesia sets in between 15 and 30 minutes on the average—in no case in less than 10 and in only a very small number of cases (5 in 397) in more than 45 minutes.

By examining single time-action curves, it was found that in dosage ranges from 5 to 15 mgm./kgm. in each *individual* rat the duration of analgesia is proportionate to the intensity reached, with a longer peak effect under higher dose. For instance, no difference in duration of analgesia was noticed among rats answering with complete analgesia in the groups treated with 5 to 6 mgm./kgm. (average duration 120 minutes on 22 rats) and 15 mgm./kgm. (average 115 minutes on 22 rats). An increase in duration was obtained, however, by inject-

⁴ "Reflex" and "Voluntary" are used empirically to designate the twitching and escape reactions. They are not intended for defining the mechanism of reaction, though it is considered possible that the twitching reaction is due to a spinal reflex.

ing a dose of 20 mgm./kgm. (average duration 230 minutes). By further investigating the dosage-duration relationship, it was found that the 20 mgm./kgm. dosage represents a ceiling dose for duration as well as for intensity. The experiment reported in table 4 shows that doses of 20 or 40 or 80 mgm./kgm. morphine all give analgesia for the same duration ($4\frac{1}{2}$ to 5 hours). It should be mentioned that when considering *average* threshold figures, the relationship between dosage and duration will still be found to be a direct one, since with higher doses a larger proportion of animals will answer with a more intense analgesia.

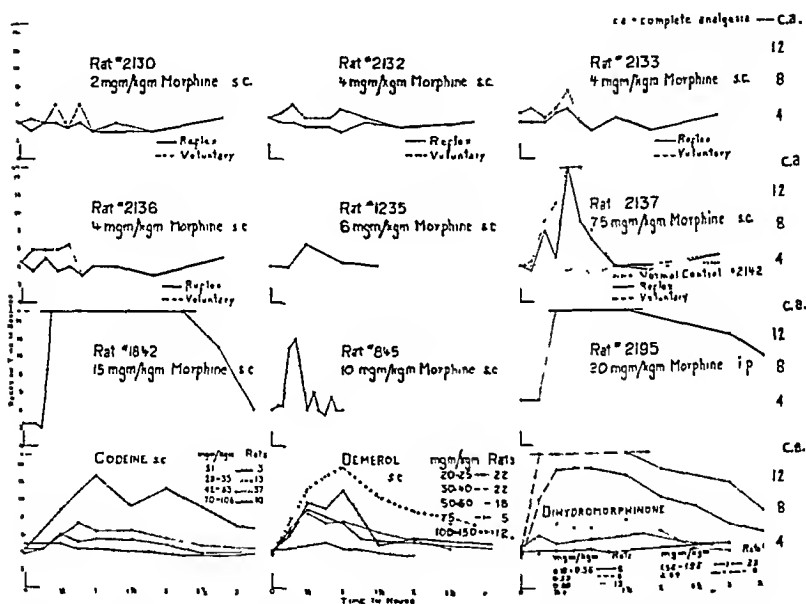


FIG. 2. Examples of individual time-action curves for morphine and average threshold variations after subcutaneous administration of codeine, demerol, and dilaudid.

(b) *Intraperitoneal injection.* Analgesia following intraperitoneal injection was less intense than when the same doses were administered subcutaneously. The M.A.D. found was 7.5 mgm./kgm.; the A.A.D., 10 mgm./kgm. Doses of 20 mgm./kgm. produced complete analgesia of three hours' duration. The type of the analgesia curve was approximately the same with both methods (fig. 2).

(c) *Intravenous injection.* Doses representing from 2 to 10 mgm./kgm. morphine base in 0.5 cc saline solution were given by intravenous injection (into tail vein). Tests for analgesia were made at close intervals (at from 1 to 5 minutes).

Of 4 rats given doses of 2 to 2.5 mgm./kgm., only one showed analgesia—and

this of late onset (29 minutes) and short duration.—In 14 rats injected with 3 to 5 mgm./kgm., there was doubtful or no response in 6; analgesia was of high degree in 2 and complete in 6.—Doses of 7.5 mgm./kgm. produced complete analgesia in 4 rats.

In general, following intravenous injection, analgesia was neither uniform nor continuous, which fact stands in contrast to the results obtained by either subcutaneous or intraperitoneal administration. In some intravenous cases, the onset of analgesia occurred after a lag period varying from 6 to 28 minutes. In others, analgesia started in less than 1 minute after injection, lasted for from

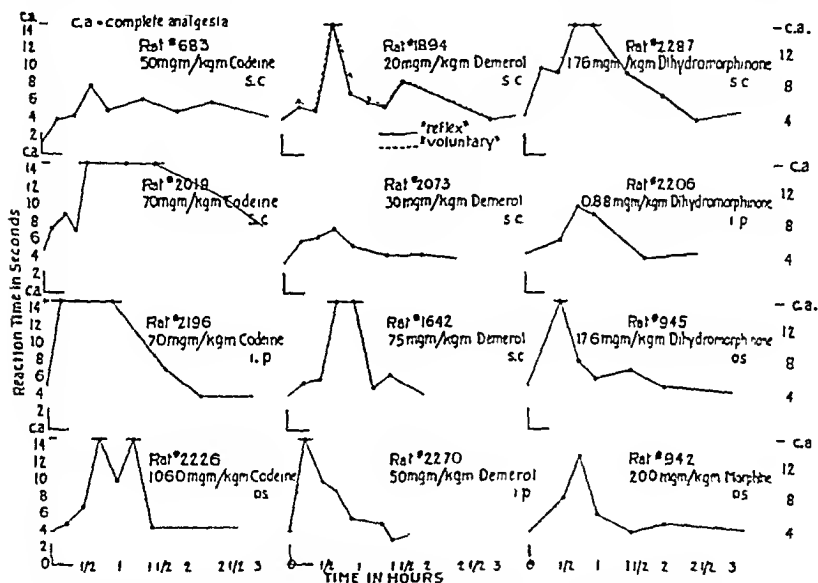


FIG 3 EXAMPLES OF TIME ACTION CURVES FOLLOWING ADMINISTRATION OF OPIATES BY VARIOUS ROUTES

3 to 10 minutes, and reappeared for 2 to 3 minutes after irregular intervals (fig 4)

(d) *Oral administration* Doses of morphine hydrochloride, corresponding to from 25 to 750 mgm /kgm base, were administered to rats orally by stomach sound. Doses ranging from 25 to 40 mgm /kgm. produced no effect in 15 rats. Out of 7 rats treated with 80 mgm /kgm., moderate analgesia of 15 to 30 minutes' duration was obtained in 3.

The average analgesic dose appeared to be between 200 and 250 mgm./kgm. Higher doses, from 300 to 750 mgm /kgm., did not produce any correspondingly greater analgesia. Such doses proved lethal.

2 THE ACTIVITY RATIOS AND THE TIME-ACTION CURVES OF MORPHINE, CODEINE, DIAMID, AND DIEMOL Supplementing our studies with morphine,

we extended the experiments to a comparison, on a quantitative basis, of the analgesic activities of codeine, dilaudid, and demerol. The time-action curves of these drugs also were determined for different routes of administration. The data gained for the analgesic behavior of demerol are of special interest, since the literature contains only a few reports on this particular phase.

Numerous investigations have been reported on the comparative analgesic values of morphine, codeine, and dilaudid. Eddy (6-8) reports extensive studies on cats in which he compared the effectiveness of the minimal analgesic dose of 1 mgm./kgm. morphine with that of 10 mgm./kgm. codeine and of 0.17 mgm./kgm. dilaudid. Oelkers (9) finds 6 to 8 mgm./kgm. of morphine hydro-

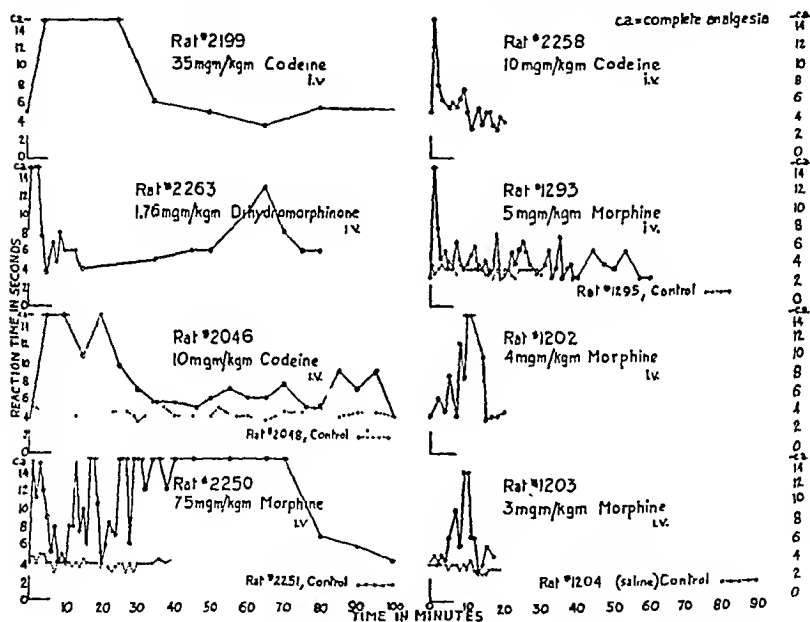


FIG. 4. EXAMPLES OF TIME-ACTION CURVES FOLLOWING INTRAVENOUS ADMINISTRATION OF OPIATES

chloride to be the dose which in mice provides a moderate degree of analgesia; of codeine, 60 to 80 mgm./kgm.; of dilaudid, 1 to 2 mgm./kgm.

According to Schaumann (10), the analgesic activity on mice of 50 mgm./kgm. of demerol is comparable to that of 7 mgm. morphine. According to a recent report by Woolfe and MacDonald (11), morphine is about seven times as active on mice as codeine and five to six times as active as demerol. The latter authors found demerol ineffective even in large doses when tested against intense pain stimuli.

Codeine. (a) *Subcutaneous injection.* Sixty-three rats were injected with doses ranging from 21 to 106 mgm./kgm. codeine-base. The M.A.D. was 28 mgm./kgm.; the A.A.D., 42

mgm./kgm. With the latter dose, analgesia set in about 30 minutes after injection and lasted for an average period of about 56 minutes. Larger doses of 70 mgm./kgm. induced complete analgesia in the majority of rats (7/8); the effect lasted for from 1½ to 2 hours.

With small doses, the peak effect was reached in less than 15 minutes after the onset of analgesia; while with higher doses (70 mgm./kgm.), it was established in from 15 to 30 minutes after onset (for averages, see fig. 2).

(b) *Intraperitoneal injection.* Twenty-two rats received intraperitoneal doses ranging from 10 to 70 mgm./kgm. Doses up to 17.5 mgm./kgm. were ineffective, while 25 mgm./kgm. induced only slight analgesia in 1 out of 5 rats. The A.A.D. was 49 mgm./kgm. In 3 rats injected with 70 mgm./kgm., complete analgesia of one hour's duration was induced (fig. 3).

TABLE 4
Analgesia obtained with 20, 40, 80 mgm./kgm. morphine s.c.

DATE, EXP NO	RAT NO	DOSE mgm / kgm	REACTION TIME (SECONDS)										
			Before	10'	20'	1 h 40'	2 h 40'	3 h 40'	4 h 30'	4 h 50'	5 h 05'	5 h 35'	24 h
4/28/43	2013	20 0	4	4.5	>15	>15		10	5	4.5	4	4	4.5
	2014		4 5	4 5	>15		>15	5	3	5	5	5	5
	2015	40 0	4	8	>15	>15		5 5	4.5	5	5	4 5	4
	2016		4	14	>15		>15	7	5 5	4	4 5	4.5	5.5
	2017	80 0	4	9	>15	>15		8	6	3.5	5	3.5	5
	2018		4 5	>15			>15	>15	8	8	5	4	5.5

TABLE 5
Average duration of analgesia in rats injected subcutaneously with various doses of dihydromorphine

DOSE DIHYDRO MORPHINE	NO RATS INJECTED	NO RATS RESPONDING WITH COMPLETE ANALGESIA	TOTAL DURATION OF ANALGESIA IN MINUTES (AVERAGE)	DURATION OF COMPLETE ANALGESIA IN MINUTES (AVERAGE)
mgm /kgm				
0 88	13	5	78	46
1 32	4	3	120	55
1 55	4	4	180	85
1.76	7	7	183	73
1 95	8	7	175	115
2 64	6	6	215	160

(c) *Intravenous injection* Twelve rats received intravenous doses ranging from 7 to 35 mgm./kgm. Repeated determinations were made at close intervals (at from 1 to 5 minutes). A dose of 10 mgm./kgm. gave high or complete analgesia in 3 out of 4 rats. It appeared one minute after injecting and lasted up to 6 minutes. Duration time was lengthened in 5 rats up to 10 minutes by the intravenous administration of 17 to 21 mgm./kgm., while 35 mgm./kgm. lengthened the duration to 25 minutes (fig. 4).

In some instances, the analgesia following intravenous injection showed an irregular and intermittent course.

(d) *Oral administration* Eleven rats received by mouth from 175 to 530 mgm./kgm.; 2 animals responded with slight and 1 with moderate analgesia; in the remaining 8, no analgesia was obtained. A dose of 1060 mgm./kgm. induced in one rat complete analgesia lasting for one hour, in another, moderate analgesia which lasted for 1½ hours (fig. 3).

Dilaudid. (a) *Subcutaneous injection.* Fifty-four rats received doses ranging from 0.18 to 2.64 mgm./kgm. Doses up to 0.36 mgm./kgm. were ineffective. The M.A.D. was found to be 0.53 mgm./kgm.; the A.A.D., 0.88 mgm./kgm. Doses higher than 1.32 mgm./kgm. (1.32 to 1.95) induced complete analgesia in over 90% of the animals (21/23). The onset of analgesia occurred in from 15 to 30 minutes, the peak being reached approximately 15 to 20 minutes later (fig. 2). Duration of analgesia lengthened as the dosage was increased (see table 5).

(b) *Intraperitoneal injection.* Determinations made on 21 rats gave the following figures: M.A.D. 0.62 mgm./kgm., A.A.D. 1.7 mgm./kgm. Complete analgesia was obtained in 3 out of 5 rats treated with 3.52 mgm./kgm., while 2 rats reacted after 7 and 12 seconds' exposure. Analgesia with the latter dosage set in 3 to 15 minutes after injection and lasted 1 to 1½ hours. The peak effect was reached 30 to 45 minutes after injection.

(c) *Intravenous injection.* Repeated tests of the state of analgesia were made at 1- to 2-minute intervals. In 2 out of 3 rats, an intravenous dose of 0.88 mgm./kgm. produced a moderate (25%) and intermittent threshold rise, while the third animal showed high, uninterrupted analgesia lasting for 10 minutes, onset occurring 1 minute after injection. In 2 rats injected with 1.32 and 1.76 mgm./kgm., respectively, there was an immediate and complete response with a duration time of 4 and 10 minutes, respectively; in 3 others, an intermittent and variable threshold rise was observed (fig. 4).

(d) *Oral administration.* A dose of 8.8 mgm./kgm. produced a slight threshold rise (15 to 20%) in 2 rats. A dose of 17.6 mgm./kgm. brought no response in 2, deep analgesia in 1, and complete analgesia in 3 rats. In 2 animals, 44 mgm./kgm. produced complete analgesia of more than 6 hours' duration.

Demerol. (a) *Subcutaneous injection.* Eighty-five rats received doses ranging from 10 to 150 mgm./kgm. The M.A.D. was found to be 20 mgm./kgm.; the A.A.D., 35 mgm./kgm. In 4 of 5 rats, doses of 75 mgm./kgm. produced analgesia of from 30 to 50 minutes' duration, with complete analgesia for 15 to 45 minutes. The onset occurred 15 to 30 minutes after injection; the peak was reached 15 to 25 minutes later. Higher doses elicited complete analgesia of still longer duration. In 4 out of 6 rats, 100 mgm./kgm. doses resulted in complete analgesia of 1 to 1½ hours' duration. In 12 rats injected with from 100 to 150 mgm./kgm., the average duration of analgesia was 138 minutes. With doses of 150 mgm./kgm., the effect was the same as with 100 mgm./kgm. (for averages, see fig. 2).

(b) *Intraperitoneal injection.* A group of 10 rats was used for injection of doses ranging from 10 to 80 mgm./kgm. The 10 mgm. dose proved ineffective in two animals. Of six animals given 50 mgm./kgm., 2 gave no response; in 2, the analgesic effect was doubtful, and 2 showed complete analgesia of 15 minutes' duration (fig. 3). In one rat, a dose of 80 mgm./kgm. induced complete analgesia of 30 minutes' duration.

(c) *Intravenous injection.* Repeated tests were made at 1- to 2-minute intervals. Three rats received 10 mgm./kgm.; two showed complete analgesia of 8 minutes' duration; the third incomplete analgesia lasting for 3 minutes with a reaction time of 8 to 10 seconds, against a normal reaction time of 4 seconds. In one rat, a dose of 20 mgm./kgm. produced complete analgesia of 13 to 14 minutes' duration. As soon as complete analgesia ceased, there followed a sudden return to the normal pain threshold.

(d) *Oral administration.* In 2 rats, 50 mgm./kgm. produced moderate analgesia of ½ and 1½ hours' duration, respectively. Four rats were given from 100 to 200 mgm./kgm. One showed complete, one high, two moderate analgesia. The duration ranged from 1½ to 2½ hours. One rat treated with 400 mgm./kgm. showed no analgesia.

3. TOXICOLOGICAL OBSERVATIONS. a) While observations were made regarding the pattern of analgesia, the appearance and nature of toxicity symptoms were also studied. The data collected are sufficiently extensive to prove that the degree of analgesia stands in no relationship to the toxic effects produced by opiates, such as temperature decrease, depression, immobility, and catalepsis.

Considerable discrepancy was noticeable between the low degree of analgesia following oral administration and the pronounced toxicity. By all methods of administration, sedation and catalepsy proved to be far more lasting than the rise in pain threshold. On the other hand, catalepsy, increase of tonus, or Straub reaction often appeared before analgesia. The following examples taken from the experimental records will illustrate these points:

Rat #950—received subcutaneously 15 mgm./kgm. morphine—catalepsy lasted for over 3 hours, analgesia for less than $1\frac{1}{2}$ hours.

Rat #2120—received orally 750 mgm./kgm. morphine. Analgesia set in $2\frac{1}{2}$ hours after administration and was of short duration; catalepsy, depression and incoordination persisted for about 4 hours. While showing a normal pain threshold, the animal died in convulsions 5 hours after treatment.

Rat #2119—received orally 500 mgm./kgm. morphine and survived treatment. Rise in pain threshold occurred $1\frac{1}{2}$ hours after treatment and was of short duration, while catalepsy lasted for more than 4 hours.

Rat #2282—Dilaudid 2 mgm./kgm. intraperitoneally. No pain threshold rise but manifest depression and incoordination of movements.

Rats #2298 and 2299—Dilaudid 4 mgm./kgm. intraperitoneally. Catalepsy 8 minutes after injection; analgesia 10 and 18 minutes, respectively.

Rat #2281—Codeine 50 mgm./kgm. subcutaneously. Incoordination, depression, Straub reaction, no analgesia.

Rats #2219 and 2220—Codeine 10 mgm./kgm. intravenously. Analgesia of 2 and 4 minutes' duration, respectively. Immobility and increased muscular tonus for 11 minutes.

Rat #2306—Demerol 100 mgm./kgm. subcutaneously. Depression, catalepsy, tail reaction appeared between 15 and 30 minutes after injection, while analgesia started 30 minutes after the disappearance of these symptoms. Tail reaction and increased tonus outlasted the analgesic effect by $1\frac{1}{2}$ hours.

b) Demerol. Toxicity determinations were made on both mice and rats. By intravenous injection, the maximum tolerated dose in mice was found to be 40 mgm./kgm.; by subcutaneous, 100 mgm./kgm. The minimum lethal intravenous dose was 70 mgm./kgm.; the subcutaneous, 175 mgm./kgm. These results agree with the mortality figures given by Schaumann (10).

For rats, the average lethal subcutaneous dose was 350 mgm./kgm. as determined on a total of 18 animals. Toxicity symptoms were similar to those following the administration of morphine: Tail reaction, increased muscular tonus, convulsions, depression, catalepsy. In some cases, doses of 150 mgm./kgm. caused hematuria.—The maximum tolerated dose by intravenous administration was 25 mgm./kgm. (4/4 survivals), the minimum lethal dose, 40 mgm./kgm. (2/5 survivals).

PART II. ANALGESIC ACTIVITY OF ACETYSALICYLIC ACID AND AMINOPYRINE. We also sought to obtain some new data on the analgesic activity of acetylsalicylic acid and aminopyrine, since the information available in the literature is rather conflicting. Hesse and co-workers (12) and likewise Hildebrandt (13) observed some analgesic influence being exerted by acetylsalicylic acid but none by aminopyrine. On the other hand, Haefliger (14) reported aminopyrine to be the only non-narcotic drug with analgesic activity. Kueter and Richards (15), and Woolfe and MacDonald (11) also observed analgesia with

aminopyrine but none with acetylsalicylic acid. Sivadjian (16) could not discover any analgesic influence of either of the two drugs.

By using the method of thermal radiation, Smith, d'Amour and d'Amour (17) were able to produce analgesia with either of the two drugs alone in oral doses of 450 mgm./kgm. rat; on the other hand, no such analgesic influence was exerted when the two drugs were administered simultaneously (aminopyrine 300 mgm./kgm. + aspirin 400 mgm./kgm.).

Our own observations were as follows:

1. Thirty-four rats were given oral doses of acetylsalicylic acid ranging from 0.5 to 2.5 gm./kgm. Judging by either the twitching or the flight reaction, the threshold of responsiveness was not raised in any of the animals. Out of 10 rats receiving doses of 2 or 2.5 gm./kgm., which is within the range of the minimum lethal dose, three died without any change in threshold level.

2. Aminopyrine was given in doses ranging from 0.3 to 1.5 gm./kgm. to 57 rats. The twitching reaction was influenced in only 20% of the animals, and

TABLE 6

Analgesia by oral administration of aminopyrine, considering twitching and flight reactions of rat

DOSE	NO. RATS	ANALGESIA IN RATS					
		Positive		Doubtful		Negative	
		Twitching	Flight	Twitching	Flight	Twitching	Flight
gm./kgm.							
0.3	3	2	3			1	
0.5-0.75	24	3	14	3	3	18	7
1.0-1.5	30	6	20	3	3	21	7
Total.....	57	11	37	6	6	40	14

here the level of response was raised only slightly (to about 20 to 40% above the normal). The threshold for the flight reaction was raised to 60 to 100% above the normal and this in 65% of the rats. As far as analgesia was concerned, no dose-effect relationship could be observed. The results are summarized in table 6.

The behavior of one rat (No. 1758) treated with 1.5 gm./kgm. aminopyrine was particularly characteristic for the difference in responsiveness evidenced in either the twitching or the flight reaction. The animal died without showing any change in the twitching reaction, while the flight threshold level was raised up to twice the normal.

Onset and duration of aminopyrine analgesia varied in different animals. Analgesia set in somewhere between $\frac{1}{4}$ to 1 hour after administration and in general lasted less than 1 hour.

DISCUSSION. Our experiments show that a suitably modified method of the Hardy-Wolff technique may be used in small laboratory animals for determining the time-action pattern of analgesics. In accordance with the findings

of these authors on humans, the reaction pattern of untreated animals is absolutely constant, and the stimulus required for pain response in treated animals stands in definite ratio to the dosage of the opiate used.

Subcutaneous administration produced the most regular and lasting effect. In general, it was more pronounced than that obtained with corresponding doses given by either intraperitoneal or oral administration.

The reaction curves following moderate or medium analgesic doses administered either subcutaneously or intraperitoneally approximate a parabola. Intravenous injection of morphine, codeine, and demerol provided a much higher intensity of analgesia than subcutaneous injection, but this was of much shorter duration.

An intermittent course of analgesia was observed in a significant number of cases after intravenous administration. Working with barbiturates, Anderson and Essex (18) reported somewhat analogous observations. When dogs were given intravenous injections of barbiturates, these authors could observe alternating disappearance and reappearance of the drug in the blood stream. No explanation of this peculiar phenomenon can be given.

After the subcutaneous administration of small analgesic doses, such an irregular course of morphine action was observed only occasionally (fig. 2).

With all methods of administration, the time curves for analgesia did not coincide with those for depression and catalepsia. As a rule, depression was of longer duration than analgesia. Following oral administration, the weak analgesic effect was particularly striking in comparison to the severity of the depression.

There exist some interesting analogies between our observations and those made on humans by Seevers and Pfeiffer (19). Determining the pain threshold by the use of the Frey-hair method, these authors found that analgesia following intravenous injection of opiates is of a lesser degree and shorter duration than that produced by subcutaneous injection. Furthermore, they observed that the degree of subjective depression and narcosis did not parallel that of analgesia. "It was a common experience for the individual to feel the greatest subjective depression following subcutaneous administration after the pain threshold had returned to its original level".

Our observation that one encounters a ceiling dose for morphine administered subcutaneously has its analogy in data obtained by clinical experimentation. Wolff, Hardy, and Goodell (3) observed that by increasing the dosage of morphine from 15 to 30 mgm, a significantly increased effect was not produced, such as could be obtained in the dosage range below 15 mgm.

The "saturation" effect described by Wolff, Hardy, and Goodell referred to intensity as well as duration of action. The fact that a dosage ceiling also exists for duration is considered by these authors to be due to a more rapid rate of elimination of the drug when the dosage is increased beyond a certain point. Our experimental results are in confirmation of this view.

By studying the data obtained by subcutaneous administration, which latter gives the most regular results, a comparison can be made of the analgesic patterns of opiates at all levels of pain relief intensity.

In contrast to previous reports on codeine (Oelkers) and on demerol (Woolfe and MacDonald), complete analgesia can be induced with these drugs when doses are sufficiently high. According to our observations with the four opiates studied, the dose required to produce complete analgesia is $1\frac{1}{2}$ to $2\frac{1}{2}$ times the average analgesic dose. Thus, it is possible to ascertain the ratios of activity at all levels of analgesia. Table 7 shows the comparative activity ratios of codeine, dilauid, and demerol in terms of morphine effectiveness.

Using pain stimuli of medium intensity, the effectiveness of codeine is found to be about one-seventh that of morphine; the effectiveness of demerol about one-sixth that of morphine. However, when checking against pain stimuli of greater intensity, the effectiveness of codeine is higher than that of demerol.⁵ In doses necessary for complete analgesia, dilauid also becomes relatively more effective.

The average analgesic doses determined by us agree fairly closely with those reported by Oelkers (9) for morphine, codeine, and dilauid, and by Woolfe and MacDonald (11) for demerol. For morphine-demerol, the ratio of effectiveness corresponds to the data reported by Schaumann (10) and those by

TABLE 7

Minimal, average, and complete analgesic doses of morphine, codeine, dihydromorphinone and demerol by subcutaneous administration (mgm./kgm.)

DOSE	MOR- PHINE	RATIO TO MOR- PHINE	CODEINE	RATIO TO MOR- PHINE	DIHYDRO- MOR- PHINONE	RATIO TO MOR- PHINE	DEME- ROL	RATIO TO MOR- PHINE
Minimal effective	4.0	1	28	1/7	0.53	7.5	20	1/5
Average analgesic	6.0	1	42	1/7	0.88	6.8	35	1/5.8
Complete analgesic	15.0	1	70	1/4.7	1.32	11.4	75-100	1/5.6

Woolfe and MacDonald (11). The minimum effective dose of codeine was found to be higher than reported by d'Amour and Smith (18 to 24 mgm./kgm. intraperitoneally), while the figure for dilauid agrees with the data reported by the two authors.

When subcutaneous doses producing the same intensity of analgesia are being compared, the curves do not differ much from each other. For instance, a 12 to 15 mgm./kgm. dose of morphine maintains analgesia for an average duration of from 114 to 149 minutes; the comparable dose of codeine, 70 mgm./kgm., for 117 minutes; of dilauid, 1.32 to 1.95 mgm./kgm., for 169 minutes; of demerol, 100 mgm./kgm., for 166 minutes. For dilauid and codeine, a quantitative relationship was observed to exist between dosage size and duration of action. The duration of analgesia following subcutaneous administration of morphine appeared to tend towards a ceiling. In *individual* rats, the duration was found to increase with the maximum intensity reached, at least with doses ranging from 5 to 15 mgm./kgm.

⁵ For codeine, the doses are expressed as free base; for demerol, as hydrochloride.

The effect produced by 150 mgm./kgm. (s.c.) demerol was not greater than that obtained with 100 mgm./kgm. Thus, the latter figure might well be considered the "maximum effective dose".—For dilaudid, the ceiling dose was not reached by injecting up to double the complete analgesic dose.

By intraperitoneal administration, morphine, dilaudid and demerol were less effective than by subcutaneous. Codeine was about equally effective by either route.

The ratios of minimum effectiveness were not the same intravenously or subcutaneously. In order to produce analgesia by intravenous administration with morphine, codeine, or demerol, approximately only $\frac{1}{4}$ to $\frac{1}{2}$ of the minimum analgesic subcutaneous dose was required, while with dilaudid approximately the same doses were required by both routes.

The oral administration gave the lowest analgesic effect. An oral dose of 200-250 mgm./kgm. morphine gave an effect corresponding to 5-7.5 mgm./kgm. given subcutaneously. An oral dose of dihydromorphinone, 17.8 mgm./kgm., produced an effect approximately equal to that of a subcutaneous dose of 0.88 mgm./kgm. With demerol, 100 to 200 mgm./kgm. orally were required to produce an effect comparable to that of 30 to 40 mgm./kgm. subcutaneously, while for codeine, over 530 mgm./kgm. per os were required to produce analgesia. No definite relationship between dose and effect could be established when opiates were given by oral administration.

The results suggest that analgesic preparations do not show uniform ratios of effectiveness by different routes of administration. This fact may well account for the difference between our results and those of Eddy and co-workers, the latter having used cats and having resorted only to intramuscular injection.

Our results indicate that acetylsalicylic acid does not influence the peripheral pain threshold in rats. This observation differs from the one reported by Wolff, Hardy, and Goodell (20) on humans. The latter authors observed that in *attentive* subjects, acetylsalicylic acid elevates the pain threshold level to 35% over the normal figure. Such an increase was elicited with 0.3 gram given *per os*, and it represents the ceiling effect that could be reached. It must be mentioned, though, that in other experiments made by the same authors (21, 22) distraction, suggestion or placebos also raised the threshold, and up to 45%. It is interesting to note that there are similarities between the graphs obtained by suggestion or placebos on the one hand and by analgesics on the other.

In view of all these factors, one may question the suitability of the Hardy-Wolff-Goodell method for the detection in humans of threshold variations lying within the range of elevation which can be brought about by suggestion.

The finding that acetylsalicylic acid exerts no influence on the *peripheral* pain threshold agrees with the common clinical observation that traumatic pain cannot be alleviated by acetylsalicylic acid.

Using the flight reaction as an indicator of pain sensation, it could be shown that aminopyrine raises the threshold level for this reaction. The twitching reaction (which can be abolished easily by small doses of opiates) was affected

In contrast to previous reports on codeine (Oelkers) and on demerol (Woolfe and MacDonald), complete analgesia can be induced with these drugs when doses are sufficiently high. According to our observations with the four opiates studied, the dose required to produce complete analgesia is $1\frac{1}{2}$ to $2\frac{1}{2}$ times the average analgesic dose. Thus, it is possible to ascertain the ratios of activity at all levels of analgesia. Table 7 shows the comparative activity ratios of codeine, dilaudid, and demerol in terms of morphine effectiveness.

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Using the flight reaction as an indicator of pain sensation, it could be shown that aminopyrine raises the threshold level for this reaction. The twitching reaction (which can be abolished easily by small doses of opiates) was affected

only very slightly even by excessively large doses of aminopyrine. Thus, *qualitatively*, the analgesic influence of aminopyrine seems to be different from that exerted by opiates. The assumption is made that twitching and flight reactions are elicited in two separate areas of the central nervous system. Both aminopyrine and the opiates influence the area responsible for the flight reaction, while only the latter drugs act at levels where the specific twitching reaction is elicited.

From a quantitative point of view, analgesia produced by aminopyrine falls far short of that produced by opiates. It was impossible to reach complete analgesia even with toxic doses, and the relative duration of analgesia was much shorter. No definite dose-effect relationship could be observed when aminopyrine was given orally. This may be attributed to the method of administration rather than to the drug itself, since with opiates, too, this relationship was not very constant.

SUMMARY

1. An adaptation of the Hardy-Wolff-Goodell method for measuring analgesia is described which makes the method suitable for pharmacological investigations on small laboratory animals. With the aid of this technique, it was possible to make some new observations and determinations, viz.:

2. Rats exhibit two reaction patterns. Both patterns are constant in untreated animals.

This observation proved useful for determining the pain threshold under varying conditions. Different doses of analgesics influenced the two reaction patterns in different degrees.

3. For purposes of exact comparison, the time-action curves of analgesia for four opiates were determined on the same species (rat) by subcutaneous, intravenous, intraperitoneal, or oral administration.—Subcutaneous administration of any of the four produced the most uniform pattern of action and an effect of longest duration. Medium size doses necessary to produce comparable intensities of analgesia show also comparable parabolic time action curves.—Intravenous injection produced analgesia of significantly shorter duration. Oral administration produced analgesia of variable duration and with high doses only.—Intraperitoneal injection of morphine, dilaudid, or demerol gave a smaller effect than subcutaneous. For codeine, the figures were approximately the same.

4. In a significant number of cases, there followed after intravenous administration a type of analgesia which showed an irregular and intermittent course.

5. For morphine, given by subcutaneous administration, the existence of a maximum analgesic dose has been ascertained. It stands at 20 mgm./kgm. rat.—With doses lower than 20 mgm., the total duration of analgesia was proportionate in general to the *individual* peak intensity reached, while the duration of the peak itself was proportionate to the dose.

6. With each of the opiates used, onset, intensity, and duration of analgesia on the one hand and depression on the other showed different time-sequences.

7. The ratios between the effectiveness of codeine, dilaudid, and demerol on the one hand and that of morphine on the other vary with the degree of pain stimulation. These ratios were established for different pain relief levels by giving the drugs subcutaneously.

8. The activity ratios between several opiates vary with the different routes of administration.

9. Aminopyrine does exert some analgesic action. This, however, is *qualitatively* different from that of the opiates.

10. The observation previously reported in the literature that acetylsalicylic acid has no analgesic activity was confirmed by our observations.

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THE PERMEABILITY OF MYCOBACTERIA TO SULFONAMIDES AND SULFONAMIDE-LIKE AGENTS

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The permeability of mycobacteria to the sulfonamides has not heretofore been demonstrated. Feinstone, et al. (1) have shown with the aid of the electron microscope that the drugs penetrate the *Streptococcus hemolyticus*, and other observers, using different techniques, have made similar conclusions. In the present report a new and simple method is described which demonstrates the permeability of mycobacteria to these and similar drugs and the extent to which it is affected by temperature, pH, drug concentration and alteration of the organism by physical agents.

The drugs used in this study were sulfathiazole (ST), sulfanilamide (SA), sulfadiazine (SD), sulfapyridine (SP), diaminodiphenylsulfone (DPS), promin and diasone.

Mycobacterium tuberculosis, Strain H₃₇, and *Mycobacterium ranae* were selected for the demonstration of permeability. They were grown on Long's synthetic medium and the experiments were carried out when the tubercle bacilli were 30 days old and the *M. ranae* 5 days old.

Bacilli grown on Long's medium produce a substance thought by some observers (2 and 3) to be para-aminobenzoic acid (PABA). This substance, whatever its true nature, gives the diazo reaction and since it occurs not only free in the medium but also in the cells its complete removal was necessary to avoid misinterpretation in subsequent color tests for the presence of the sulfonamides. For this reason the following thorough washing procedure was employed prior to treatment with the sulfonamides.

Approximately 10 grams of a wet culture of mycobacteria were placed in a Buchner filter and freed of medium by thorough washing with distilled water. The bacilli were then placed in 200 ml. of distilled water and incubated for 24 hours at 37°C., the flask being agitated occasionally. The bacillary suspension was then filtered and the process repeated until the organisms were free of the diazo-reacting substance.

After treatment with the sulfonamides in the various ways described in the individual experiments the organisms were repeatedly washed in distilled water and centrifuged until the supernatant fluid and the sedimented cells showed no color reaction to the Bratton and Marshall test. The color reactions were read as + + + +, + + +, + +, and +, which are approximately equivalent to color standards made up with 0.4 mg.%, 0.3 mg.%, and 0.2 mg.% and 0.1 mg.% respectively of sulfathiazole.

EXPERIMENT 1. *Influence of temperature and concentration of ST on perme-*

ability. Washed *M. ranae* were placed in distilled water containing 5 mg.% and 25 mg.% of ST (2-sulfanilyl aminothiazole, Squibb). One half of each suspension was incubated at 37°C. for 48 hours and the other at 10°C. for 48 hours. The bacilli were then repeatedly washed with distilled water until no color reaction was produced with the Bratton and Marshall method.¹ Three washings were required for the 5 mg.% concentration and five for the 25 mg.% concentration. The sedimented bacilli were then dried in the incubator and ground in a mortar until the material was no longer acidfast. 100 mg. of the ground bacilli were suspended in 1 ml. of distilled water and tested for ST. Tubercle bacilli (Strain H₃₇) were also treated in this manner. The results are presented in table 1.

From the table it may be seen that only the crushed bacilli gave a color reaction and that the deepest color was obtained with the higher concentration of the drug. The tubes containing the tested crushed bacilli were centrifuged and it was observed that color was present in the sedimented portion only. Binding of the drug was more pronounced at 37°C. than at 10°C. as indicated by color intensity. *M. ranae* and H₃₇ reacted similarly with regard to crushing

TABLE 1
Sulfathiazole

ORGANISM	37°C		10°C.	
	25 mg. %	5 mg. %	25 mg. %	5 mg. %
H ₃₇ intact	0	0	0	0
H ₃₇ crushed	++++	++	+	0
<i>M. ranae</i> intact	0	0	0	0
<i>M. ranae</i> crushed	++++	++	+	0

temperature and concentration of the drug. Untreated intact and crushed bacilli, used as controls, were negative to the diazo reaction.

EXPERIMENT 2. *Rate of extraction of ST from bacilli at different temperatures.* Since the crushed, but not the intact, bacilli showed the presence of ST it seemed reasonable to assume that the crushing made it possible to determine the presence of the drug. This experiment was set up for the purpose of extracting the drug from the intact cell.

The intact mycobacteria from Experiment 1 which had been treated with 25 mg.% ST and washed thoroughly were divided into three portions, each weighing approximately 500 mg. (wet weight). The portions were placed in separate test tubes containing 5 ml. of distilled water and the contents thoroughly mixed. The tubes were incubated for 24 hours at 37°C., 22°C., and 10°C. respectively. The supernatant fluid was then decanted and evaporated to 1 ml. volume and when tested for ST gave the following results: at 37°C. +++++; at 22°C. ++; and at 10°C. +. 5 ml. of water were then added to the sediment in each tube

¹ Since small amounts of material were tested, correspondingly small amounts of the reagents for B and M's test were used—0.3 ml. of each proved satisfactory.

and at this time all the tubes were incubated at 37°C. for 24 hours. The supernatant fluid was again decanted and evaporated to 1 ml. volume. The tube previously incubated at 37°C. now showed no evidence of ST, that at 22°C. ++, and that at 10°C. +++. The bacilli from all the tubes were then separately dried and crushed and by B and M's test were free from ST.

This experiment indicates that the drug in the intact bacillus can be withdrawn in the presence of water and that the rate of withdrawal is more rapid at higher temperatures. Untreated washed bacilli, used as controls, were extracted at 37°C. for 48 hours. The concentrated supernatant fluid failed to give the diazo reaction.

EXPERIMENT 3. *Extraction of ST from crushed bacilli at different temperatures.* In the following experiment *M. ranac* was treated with 25 mg.% ST in water for 48 hours at 37°C. After the removal of free ST by washing, the organisms were dried and crushed. Three 100 mg. portions were placed in 5 ml. of water each, shaken thoroughly and incubated for 24 hours at 37°C., 22°C., and 10°C., respectively. The supernatant fluid was evaporated to 1 ml. as before and tested for ST. The tube incubated at 37°C. showed ++++ color, that at 22°C.+++ and the 10°C. sample ++. Fresh water was added to the sediment and all tubes re-incubated at 37°C. for 24 hours. The 37°C. sample was negative and the 22°C. and 10°C. samples gave + and ++ respectively. The sediment was also tested and showed no detectable color.

Experiments 2 and 3 are in general comparable. The only significant difference was the increased rate of extraction of the crushed bacilli at the lower temperatures. This might be due to the fact that during the process of crushing the cell was broken, thus facilitating the extraction process.

EXPERIMENT 4. *Period required for the maximum penetration of ST.* Ten grams (wet weight) of washed *M. ranac* were placed in an Erlenmeyer flask containing 25 mg.% ST in 200 ml. of distilled water and incubated at 37°C. At intervals of 2, 6, 12, 24, 48 hours and 4 days samples were removed and the cells washed in distilled water until no color reaction was obtained. 500 mg. of sedimented bacilli from each sample were extracted in the usual manner and the following results were obtained: 2 hours +, 6 hours ++, 12 hours +++ and the others +++. To each tube of the series 5 additional ml. of water were added and the extraction continued for 24 hours. No trace of ST was found in any of the tubes except in the 48 hour and 4 day samples and here only a trace of the drug was found.

It may be concluded from the above experiment that it requires about 48 hours for the cells to take up a maximum amount of the drug.

EXPERIMENT 5. *Rate of extraction of ST from M. ranac.* *M. ranac* was treated with 25 mg.% ST for 48 hours and washed free of the drug. 500 mg. (wet weight) of the cells were placed in 5 ml. of distilled water and after brief shaking the cells were thrown down by centrifugation. The concentrated supernatant fluid was negative for free ST.

5 ml. of water were added to the sediment and the tube incubated at 37°C.

for one hour. This one hour extraction showed a trace of color by B and M's test. The procedure was repeated by adding fresh 5 ml. amounts of water to the same sediment and incubating for periods of 5 hours, 20 hours, and 24 hours. The concentrated supernatant fluids were tested after each incubation period and the results were as follows: 5 hours ++, 20 hours ++ and 24 hours no detectable color.

When *M. ranae* was treated with ST prepared in isotonic sodium chloride solution the results were comparable to *M. ranae* treated with water solutions of ST. However, 48 hours at 37°C. were required to extract all of the drug present in the bacilli as compared to 24 hours in distilled water.

5% hypertonic salt solution (NaCl) containing 25 mg.% ST was used in like manner. Although more washings were required to remove free ST from the supernatant fluid and sediment, the results were similar to those obtained with physiological saline.

The viability of *M. ranae* was not affected by this treatment.

EXPERIMENT 6. *Permeability of M. ranae to related drugs.* *M. ranae* treated with other compounds (SP, SD, SA, DPS, promin, diasone and diasone plus sodium bicarbonate) in the usual manner yielded results qualitatively similar to but quantitatively different from the results obtained with ST. This was due probably to the difference in the amount of color developed by the various agents in response to Bratton and Marshall's test.

The most striking results were obtained with SA, DPS and diasone, all of which required additional washings to remove free drug and produced an intense color reaction on extraction.

EXPERIMENT 7. *Effect of pH on permeability.* In this experiment the effect of ST in buffer solutions of varying pH values was studied. 25 mg.% solutions of the drug were made at pH values of 4.0, 7.0 and 9.0, and washed *M. ranae* were added. The bacilli were treated as in the preceding experiments until the supernatant fluid and the sediment showed no free ST by the B and M method. 500 mg. (wet weight) of the bacilli from each pH value were placed in 5 ml. of water and incubated at 37°C. for 24 hours. The supernatant fluid was concentrated and tested for free ST. The results were: pH 4.0 +++, pH 7.0 +++, and pH 9.0 a trace of color. Water was again added to the sediment and after 24 hours no further color was obtained.

This experiment was repeated with the tubercle bacillus and a comparison of the findings is presented in table 2.

EXPERIMENT 8 *Permeability of sulfonamide-resistant M. ranae.* The purpose of this experiment was to determine the permeability of *M. ranae* rendered resistant to ST. The organism was cultivated on Long's medium in graded concentrations of ST until a luxuriant growth was obtained in a concentration of 100 mg.% whereas originally growth was inhibited by 1 mg.% of the drug. The resistant organism was then grown for ten generations on Long's medium free of the drug, and at this time it was found that no diminution had occurred in the resistant properties of the organism. These resistant organisms produced

approximately ten times as much of the diazo-reacting substance in Long's medium as did the susceptible parent strain, and as a consequence, required additional washing prior to treatment with ST.

The washed bacilli were placed in 25 mg.% ST for 48 hours at 37°C. They were then thoroughly washed and the ST extracted as outlined in previous experiments. The rate and amount of extraction of ST was the same for the resistant as for the sensitive organisms.

This ST-resistant *M. ranae* was also fast to the other sulfonamide compounds used in this study. The permeability of the resistant organisms to these drugs was also tested and the results were not appreciably different.

EXPERIMENT 9. *Permeability of dead cells to ST.* It has been established that there is a marked difference in permeability between the living and the dead cell (4) which is confirmed by the following experiment.

M. ranae were placed in 10% formalin for 1½ hours prior to treatment with ST. It was found that after ten washings there was still a suggestion of color in the supernatant fluid and the sediment gave an intense color reaction. Ex-

TABLE 2
Sulfathiazole

ORGANISM	DURATION OF EXTRACTION	COLOR INTENSITY AT		
		pH 4.0	pH 7.0	pH 9.0
<i>M. ranae</i>	24 hours	+++	++++	trace
	48 hours	0	0	0
<i>M. tuberculosis</i> Strain H ₃₇	24 hours	++++	++++	++++
	48 hours	+	+	++
	3 days	0	0	0

traction of 500 mg. (wet weight) of the washed cells was carried out for six consecutive days and at this time the supernatant fluid still gave a marked color reaction. Tubercle bacilli treated with formalin behaved in a similar manner.

The same procedure was carried out with heat-killed *M. ranae* and with *M. ranae* rendered non-acidfast by treatment with Aronson's solution (ether-alcohol 1:1 + 1% HCl) for five days. After treatment with ST it was found that the heat-killed and non-acidfast forms contained less ST than the organisms killed by formalin but considerably more than the live organisms, as determined by Bratton and Marshall's test.

The experiment shows that dead *M. ranae* retain considerably more of the drug than do the live bacilli.

DISCUSSION. The evidence presented in the first six experiments described in this paper indicates that sulfonamides and sulfonamide-like agents are bound to mycobacteria both outside (on the surface of the bacterial cell) where the drug presents a free para-amino group with which it can be diazotized, and within the cell where it cannot be demonstrated until the cell is mechanically

crushed. It has also been demonstrated that this binding of the drug with loci on and in the bacterial cell is dependent upon time and temperature. Davis (5) demonstrated in his work on the binding of sulfonamides to proteins that there was practically no difference between the degree of binding at 5° and at 37° in 24 hours, whereas in these experiments there was a marked difference, indicating that a relatively large amount of the sulfonamide enters the bacterial cell and that time and temperature are important factors in determining this amount. Consideration of the relatively slow rate at which the agent enters the cell may explain, in part, the lag period which has been observed in bacteriostasis of microorganisms by sulfonamides.

Another point of interest in the observations presented here is that sulfanilamide is taken up by the bacterial cells in larger amount than is sulfathiazole (even when consideration is taken of the fact that a somewhat smaller amount of this agent, approximately two-thirds, gives a color reaction by the B & M test equivalent to that of sulfathiazole). If the amount of a sulfonamide taken up by a bacterial cell were simply a function of the degree of binding of that sulfonamide to protein, one would expect on the basis of the observations of Davis (5) that only one-sixth as much sulfanilamide as sulfathiazole would be taken up by the cell at pH 7.0. However, when one assumes that the amount of sulfonamide which penetrates the peripheral structures of the bacterial cell is to a large extent a function of the concentration of the unionized species of the agent, as has been recently suggested (6), these observations are readily explainable, for sulfathiazole is much more ionized at pH 7 than is sulfanilamide.

As to the important question, whether or not sulfonamides and sulfonamide-like agents must enter the bacterial cell in order to effect bacteriostasis, the observations presented in this paper do not give a definite answer. Various observers have emphasized the importance of the physico-chemical state of electro-negativity of the SO_2 group as the primary determinant of bacteriostatic activity of these agents (5, 6, 7) and related this to the binding of the drugs to plasma proteins (5) and the effects of the sulfonamides on the electrokinetic mobility of bacterial suspensions (8), and it has not been unequivocally demonstrated that bacteriostatic agents which antagonize para-aminobenzoic acid must pass through the peripheral structures of microorganisms in order to reach the locus at which they exert their specific metabolic effect.

The experiment with sulfonamide-fast organisms significantly demonstrates that the development of sulfonamide-resistance is accompanied by increased production by the bacteria of a substance or substances which give the diazo reaction, and that the acquisition of sulfonamide-resistance does not measurably affect the permeability or the capacity of the organisms to take up sulfonamides from a solution as observed by means of the technique described.

The well known freeing of acidic groups on death of bacterial cells may account for the observed increase in binding of sulfathiazole by killed mycobacteria inasmuch as more groupings would thus be made available in and on the bacterial cells for coupling with the basic amine group of the sulfonamides. But also, in view of the greater permeability of the peripheral structures of dead

cells, a frequently observed phenomenon, this increased binding may be due to the non-selective passage of both the unionized and the ionized forms of sulfathiazole into the bacterial cell, contrasting with the more or less selective passage of only the unionized form of low molecular weight organic compounds into living cells (4).

SUMMARY

Evidence has been obtained by means of a newly described technique that sulfonamides and sulfonamide-like agents enter mycobacterial cells and the possible relation between this fact and the mechanism of bacteriostasis by these agents is discussed.

Sulfonamide-fast mycobacteria produce large amounts of diazotizable material and the development of drug-fastness does not appear to depend upon the change in permeability or binding of the sulfonamide to the bacterial cells.

Mycobacteria killed by various techniques are demonstrated to bind more sulfonamide than do living organisms and the possible significance of this observation is discussed.

We wish to express our thanks to Dr. René J. Dubos and Dr. Gardner Middlebrook for their helpful suggestions.

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STUDIES ON SHOCK INDUCED BY HEMORRHAGE

XII. SOME PROPERTIES OF A NATURALLY OCCURRING INHIBITOR OF *D*-AMINO ACID OXIDASE¹

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INTRODUCTION. A number of naturally occurring anti-enzymes and enzyme inhibitors have been reported in the literature. These inhibitors exhibit widely differing properties and exert their inhibitions by a variety of mechanisms. One of the first inhibitors to be investigated was probably anti-catalase, which was found by Battelli and Stern (1) to be a heat stable, alcohol soluble substance which oxidised catalase.

Inhibitors of proteolytic enzymes have been found. Northrup (2) isolated trypsin inhibitor, and Herriott (3) crystallized pepsin inhibitor. Both of the latter had the properties of polypeptides.

Inhibitors which act by hydrolysing the coenzyme are also known. Ochoa (4) found that under anaerobic conditions cocarboxylase was hydrolysed enzymatically. Cozymase and alloxazine adenine dinucleotide are destroyed by nucleotidases present in tissues (5, 6, 7) and glutathione, the coenzyme of glyoxalase is hydrolysed by an enzyme, antiglyoxalase (8).

Geiger (9) has demonstrated the presence of a glycolytic inhibitor in brain and Krebs (10) found evidence for an inhibitor of *D*-amino acid oxidase in kidney. Neither of these factors was further investigated.

In a preliminary communication (11) we reported that anoxia decreased the ability of excised liver to metabolise lactic and amino acids. This was assumed to be caused by the presence of some substance in the tissue which became active under anaerobic conditions. On adding a water extract of liver to the freshly excised tissue or to the isolated *D*-amino acid oxidase, a similar decrease in the metabolism of these substrates was produced. It was found in these experiments in the case of *D*-amino acid oxidase that the inhibitor did not hydrolyse the coenzyme but appeared to produce its effect by acting on the apoenzyme, or protein part of the enzyme.

The present communication describes the partial purification, properties and conditions for activity of the inhibitor as well as the kinetics of its action.

EXPERIMENTAL Methods Oxygen consumption was measured in Warburg manometers at 37°C. Usually each vessel contained a total volume of 2 cc. in the main chamber; 0.3 cc 8% KOH and a roll of filter paper were placed in the center well to absorb CO₂.

Nitrogen was determined by the micro Kjeldahl method using Ma and Zuazaga's modifications (12). Protein was precipitated with metaphosphoric acid, centrifuged and di-

¹ This work was carried out under a grant from the Mallinckrodt Chemical Works

gested in the usual manner. When non-protein nitrogen determinations were desired an aliquot of the supernatant fluid was used.

d-Amino acid oxidase was prepared from sheep kidneys by the method of Warburg and Christian (13). Purification was carried only as far as the third $(\text{NH}_4)_2\text{SO}_4$ precipitation. It was then suspended in pyrophosphate buffer and kept frozen in solid CO_2 . 0.2 cc. was used in each vessel. Under optimum conditions this amount of enzyme with excess allo-vazine adenine dinucleotide and substrate absorbed about 600 mm.³ O_2 per hour. Allovazine adenine dinucleotide (AAD) was prepared from Fleischmann's bakers' yeast by the method of Warburg and Christian (13), and its purity, determined from the data of these workers, was found to be 0.042.

Partial purification of the inhibitor. Rat, dog, and beef livers were used as sources of inhibitor. One part of liver by weight was ground with four parts of distilled water. Small amounts of tissue were ground in a modified Potter and Elvehjem homogenizer (14) obtained from the Scientific Glass Apparatus Company; larger amounts were finely divided in the Waring Blendor. Freezing the liver prior to extraction with water or freezing the water suspension often increased the activity of the preparation. After centrifuging, the supernatant fluid exhibited activity almost equal to that of the original suspension, and for this reason the solid material was discarded. To the supernatant fluid an equal volume of citrate-phosphate buffer of pH 3.0 was added (4.11 parts 0.2M Na_2HPO_4 and 15.89 parts 0.1M citric acid) (15). The mixture was again centrifuged and the inactive precipitate was discarded. The pH of the supernatant fluid was adjusted to 4.95 with 10% NaOH and the small amount of inactive precipitate which formed was discarded. To the clear, red fluid an equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$ was added, the mixture was centrifuged, and the precipitate suspended in distilled water. Occasionally complete solution was obtained, but more often a portion of the precipitate remained insoluble. The suspension was dialysed overnight in running tap water to remove the remaining $(\text{NH}_4)_2\text{SO}_4$. The precipitate which remained after dialysis was separated by centrifugation and washed three times with small amounts of water. The pooled clear solutions were quite active. Precipitation with $(\text{NH}_4)_2\text{SO}_4$ after adjusting the pH to 7 resulted in preparations having very low activity.

$(\text{NH}_4)_2\text{SO}_4$, MgSO_4 and NaCl in various concentrations were used as precipitating agents. Some active material could be precipitated by 30% saturation with $(\text{NH}_4)_2\text{SO}_4$. Full saturation with $(\text{NH}_4)_2\text{SO}_4$ did not increase the yield over that obtained with half saturation. Full saturation with MgSO_4 or NaCl would also precipitate the inhibitor.

The inhibitor could be further purified by adsorption on and elution from $\text{Cu}(\text{OH})_2$, but large losses of material were experienced. The $\text{Cu}(\text{OH})_2$ was prepared by the method of Herriott (3). Both phosphate buffer pH 7.8 and Na_2HPO_4 in concentrations of M/10 and M/2 were used to elute the protein from the $\text{Cu}(\text{OH})_2$. Phosphate buffer pH 7.8 M/2 appeared to give the best results. After elution from the $\text{Cu}(\text{OH})_2$ and centrifugation, the inhibitor was precipitated by half saturation with $(\text{NH}_4)_2\text{SO}_4$, centrifuged and dissolved in water. The fractionations described were carried out at room temperature but all solutions were kept on ice when not in the centrifuge. The solutions were dialysed against tap water to remove the precipitating agents.

Table 1 gives the activities and protein N contents of the various fractions.

Since the activity exhibited by the inhibitor is not directly proportional to the concentration, various dilutions were tested. It will be seen from table 1 that the maximum activity per mg. N is reached with the $\text{Cu}(\text{OH})_2$ eluate. In several experiments comparable inhibitions were obtained with $\text{Cu}(\text{OH})_2$ eluates containing approximately the same amount of protein N.

Stability of the extract. The inhibitor, whether in the intact liver or in water extract is stable to freezing (table 2). Freezing the whole liver appears to increase the yield of inhibitor, probably by increasing the disruption of the cells and thus liberating the active factor to a greater extent.

TABLE 1
Activities and protein N contents of various liver fractions

FRACTION	PROTEIN N/VESSEL	ACT. O_2		INHIB.	N TO PRODUCE 100% INHIB.
		Boiled extract	Active extract		
	mg			%	mg.
Original liver, 1 gm. in 10 cc	1.11	805	458	43	2.57
After precipitation at pH 4.0	0.588	496	318	36	1.63
$(\text{NH}_4)_2\text{SO}_4$ precipitate, fraction I	0.1176	750	498	34	0.345
$(\text{NH}_4)_2\text{SO}_4$ precipitate, fraction II	0.495	910	500	45	1.10
$(\text{NH}_4)_2\text{SO}_4$ precipitate, fraction III	0.2136	868	481	45	0.475
$(\text{NH}_4)_2\text{SO}_4$ fraction	1.5	540	109	84	1.78
	0.5	540	202	64	0.775
Various dilutions	0.166	540	299	44	0.377
	0.055	540	361	35	0.157
	0.0183	540	403	27	0.0678
$\text{Cu}(\text{OH})_2$ eluate	0.0639	323	180	44	0.145
	0.0319	323	209	35	0.091
Various dilutions . . .	0.0159	323	229	29	0.0548
	0.0079	323	221	32	0.0247
	0.0039	323	231	29	0.0135

All vessels contained 0.2 cc. enzyme, 0.2 cc. 4.5% *D,L*-alanine, 0.2 cc. tissue extract, 0.1 cc. *AD* (1 mg./cc.) and 1.3 cc. buffer.

Heating for 5 minutes at 60°C. partially inactivates the inhibitor and heating at 100° for 5 minutes results in complete inactivation.

Evaporating to dryness at room temperature in air inactivates the extract whereas evaporation under the same conditions to a small volume does not affect the activity (table 2).

Effect of buffer concentration on the activity of the inhibitor. Table 3 shows that with increased concentration of phosphate there is a decrease in the activity of the inhibitor. The per cent inhibition produced in M/10 phosphate buffer is considerably less than that in M/60 buffer. In three different experiments the inhibition produced in M/10 phosphate was 39, 28 and 27%, whereas in M/60 phosphate the corresponding inhibitions were 46, 49 and 49%.

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TABLE 2

*Effect of freezing, heating and drying on the activity of the extract**O₂ uptake of the d-amino acid oxidase system with and without extract*

	O ₂ UPTAKE	INHIBITION
	mm. ³	%
1. Without extract.....	605	
Extract.....	450	26
Extract frozen and thawed.....	380	37
Extract made from liver previously frozen.....	361	40
2. Without extract.....	612	
Extract heated at 60° for 5 min.....	575	6
Extract heated at 100° for 5 min.....	621	0
3. Without extract.....	612	
Extract.....	415	31
Extract evaporated to dryness at room temperature...	618	0
4. Extract boiled	618	
Extract	493	20
Evaporated extract diluted to same concentration as above—boiled.....	653	
Evaporated extract diluted to same concentration as above.....	540	17

In experiments 1 and 2, water extracts of whole liver were used; in experiments 3 and 4, extracts purified by ammonium sulphate precipitation were used. Cu(OH)₂ cluates were partially evaporated in other experiments without loss of activity.

TABLE 3

Effect of phosphate concentration on the activity of the inhibitor

1.1 cc. of buffer at pH 7.2 and at the molarity indicated was added to each vessel. Final volume was 2 cc.

	MM. ³ O ₂ ABSORBED			
	M/10	M/30	M/60	M/120
No extract.....	529	617	594	617
Extract.....	323	340	321	308
% inhibition.....	39	45	46	48
No extract.....	390		440	
Extract.....	279		225	
% inhibition.....	28		49	
No extract.....	384		421	
Extract.....	279		213	
% inhibition.....	27		49	

Effect of pH. From table 4 it may be seen that by decreasing the pH of the medium an increase in inhibition produced by the extract results. *d*-Amino acid oxidase exhibits its greatest activity at pH 9. As the pH is lowered its activity decreases. In the presence of inhibitor, however, the decrease in activity with change in pH is seen to be greater. At low pH the extract exerts its greatest activity, and the per cent inhibition is greatest.

Possible proteolytic action of the inhibitor on the apoenzyme. In our previous communication we showed that the inhibitor seemed to exert its action by attacking the apoenzyme or protein part of the enzyme and not the coenzyme. It seemed possible that the inhibitor might be a cathepsin and exert its activity by

TABLE 4

Effect of pH on the activity of the inhibitor of d-amino acid oxidase

All vessels contained 1.1 cc M/60 buffer in a total volume of 2 cc.

	pH	mm ³ O ₂ ABSORBED AT VARIOUS pH'S				
		5.3	5.9	7.0	7.6	9.3
Experiment I						
No extract		406	386(?)	467	530	552
Extract		150	146	220	268	275
% inhibition		63	62	53	50	50
	pH	5.6	7.1			
Experiment II						
Boiled extract		190	632			
Active extract		85	403			
% inhibition		55	36			
	pH	6.4	7.4			
Experiment III						
Boiled extract	...	245	377			
Active extract		145	240			
% inhibition	.	41	36			

hydrolysing the apoenzyme. This possibility was tested by incubating the apoenzyme with the inhibitor under the usual conditions of the experiment and determining NPN and protein N at intervals. No change in either protein N or NPN could be detected during the course of one hour by the methods used.

Reversibility of inhibitory reaction. Since the per cent inhibition decreases with increase in pH of the medium it was thought that this fact might be used to test the reversibility of the reaction. Experiments were, therefore, set up at two considerably different pH's and O₂ consumption was measured in the presence and absence of inhibitor. The pH of the reaction carried out at low pH was then raised to that of the reaction carried out at the higher pH and O₂ consumption again measured. It was found that the per cent inhibition decreased when the

pH was raised. The results of one typical experiment are given in table 5 and figure 1. Here it may be seen that in the vessel which was maintained at a low pH during the whole experimental period the inhibition was 53% for the first half of the experimental period and 58% for the second half, whereas in the vessel in which the pH was raised from 5.6 to 7.2 the per cent inhibition decreased from 52 to 36% (average of duplicate vessels). This would seem to indicate that by increasing the pH of the medium the inhibitor can be dissociated from the enzyme.

Kinetics of inhibition. Since the action of the inhibitor appears to be reversible it seemed to be of interest to determine:

- (1) whether the inhibitor combined with the apoenzyme and competed with the coenzyme for the apoenzyme.

TABLE 5

Reversibility of inhibitory action

All vessels contained 0.2 cc. enzyme, 0.1 cc. AAD, 1.0 cc. buffer, 0.2 cc. alanine and 0.3 cc. extract. The side bulb contained 0.06 cc. M/2 buffer. O₂ consumption was measured for 40 minutes before the liquid in side bulb was tipped in and for a further 40 minutes afterwards.

pH at start	7.3	5.6	5.6	5.6
Boiled extract (mm. ³ O ₂) . .	333	101	106	125
Active extract (mm. ³ O ₂) . .	196	48	55	55
% inhibition.	24	53	48	56

Addition from side bulb				
	BUFFER 7.4	BUFFER 5.15	Na ₂ HPO ₄	Na ₂ HPO ₄
pH after addition	7.3	5.8	7.1	7.2
Boiled extract (mm. ³ O ₂) . .	299	89	254	383
Active extract (mm. ³ O ₂) . .	207	37	170	171
% inhibition	32	58	33	39

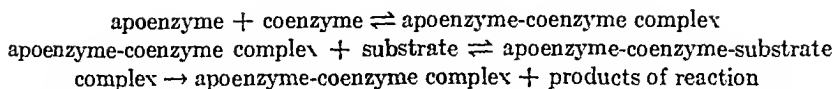
- (2) whether it combined with the whole enzyme (apoenzyme-coenzyme complex).

- (3) whether the inhibitor competed with the substrate for the enzyme-coenzyme complex.

Michaelis and Menten (16) proposed that an enzymatic reaction proceeds by way of an enzyme-substrate intermediate which breaks down to give the products of the reaction:

Enzyme + substrate \rightleftharpoons enzyme-substrate complex \rightarrow products of reaction + enzyme. The reaction velocity is then assumed to be proportional to the concentration of the enzyme-substrate complex.

Stadie and Zapp (17) applied this concept to the catalytic action of an enzyme which is dependent on a coenzyme for its activity and assumed the following equilibria to exist:



Their experimental results agreed with the theoretical considerations based on equations expressing velocity in terms of equilibrium constants.

Lineweaver and Burk (18) have considered various aspects of the Michaelis-Menten theory especially as it applies to reactions in which different types of inhibitors are present. When the reciprocal of the velocity is plotted against

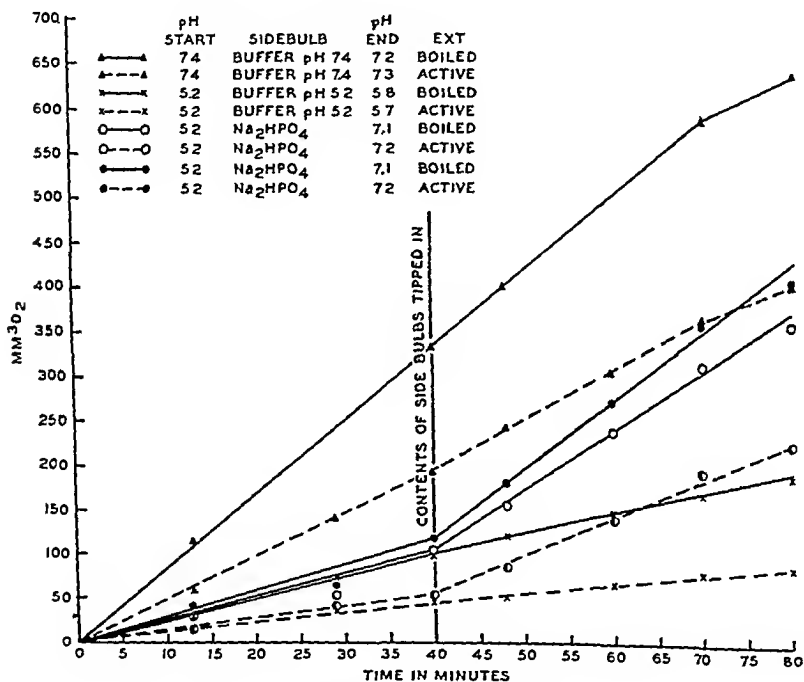


FIG 1 REVERSIBILITY OF INHIBITORY ACTION.
See table 5 for explanation

the reciprocal of the substrate concentration straight lines are obtained both in the presence and absence of inhibitor. If the inhibitor competes with substrate for the enzyme, the lines obtained in the presence and absence of inhibitor have a common intercept, but in the presence of inhibitor the slope is increased. If the inhibition is noncompetitive, the intercept is increased, but the slopes remain equal. The derivation of the equations used to represent the velocity-substrate relationships in the presence and absence of such inhibitors are given by Lineweaver and Burk (18), and by Wilson (19, 20).

We have developed equations to represent conditions in which an inhibitor for *d*-amino acid oxidase is present, and applied them to the interpretation of experimental results for determining the mechanism of action of the inhibitor.

Symbols. The symbols are similar to those used by Stadie and Zapp (15) except that we use the term AAD for the coenzyme in conformity with our previous publications.

v = velocity of the reaction in micromoles/min.

(P) = concentration (mole/l.) total enzyme

(p) = concentration (mole/l.) of protein not combined with coenzyme or amino acid

(A) = concentration (mole/l.) of total AAD

(a) = concentration (mole/l.) of free AAD

(S) = concentration (mole/l.) of *d*(-)-amino acid

(I) = concentration of inhibitor

(PA) = concentration (mole/l.) of protein-AAD complex

(PAS) = concentration (mole/l.) of protein-AAD-amino acid complex

(PI) = concentration of protein-inhibitor complex

(PAI) = concentration of protein-AAD-inhibitor complex

K_{PA} = dissociation constant of protein-AAD complex

K_{PAS} = dissociation constant of protein-AAD-amino acid complex

K_{PI} = dissociation constant of protein-inhibitor complex

K_{PAI} = dissociation constant of protein-AAD-inhibitor complex

k = proportionality constant

1. *Competition between inhibitor and AAD.* Stadie and Zapp (17) have shown that for the amino acid oxidase system incompletely saturated with coenzyme the following relationship exists:

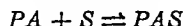
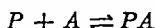
$$\frac{1}{v} = \frac{1}{k(P)} \left[\left(1 + \frac{K_{PAS}}{(S)} \right) + \frac{K_{PAS} \cdot K_{PA}}{(S)(a)} \right]$$

and when $\frac{1}{v}$ is plotted against $\frac{1}{(a)}$ a straight line results having

an intercept of $\frac{1}{k(P)} \left(1 + \frac{K_{PAS}}{(S)} \right)$ and a

slope of $\frac{1}{k(P)} \cdot \frac{K_{PAS} \cdot K_{PA}}{(S)}$

If the inhibitor competes with the coenzyme it probably combines with the enzyme by the same mechanism as does AAD. In the presence of inhibitor which competes with the coenzyme the following equilibria are assumed to exist.



$$(P) = (p) + (PA) + (PAS) + (PI) \quad (1)$$

$$(A) = (a) + (PA) + (PAS) \quad (2)$$

$$K_{PA} = \frac{(p)(a)}{(PA)} \quad (3)$$

$$K_{PAS} = \frac{(PA)(S)}{(PAS)} \quad (4)$$

$$K_{PI} = \frac{(p)(I)}{(PI)} \quad (5)$$

Combining equation 1, 3, 4 and 5,

$$\begin{aligned} (P) &= \frac{K_{PA}(PA)}{(a)} + \frac{K_{PAS}(PAS)}{(S)} + (PAS) + \frac{(p)(I)}{K_{PI}} \\ &= (PAS) \left[\frac{K_{PA} \cdot K_{PAS}}{(a)(S)} + \frac{K_{PAS}}{(S)} + 1 + \frac{K_{PA} \cdot K_{PAS}(I)}{(a)(S)K_{PI}} \right] \end{aligned}$$

If $v = k(PAS)$,

$$\frac{1}{v} = \frac{1}{k(P)} \left[1 + \frac{K_{PAS}}{(S)} + \frac{1}{(a)} \left(\frac{K_{PA} \cdot K_{PAS}}{(S)} + \frac{K_{PA} \cdot K_{PAS}(I)}{(S)K_{PI}} \right) \right]$$

Intercept = $\frac{1}{k(P)} \left(1 + \frac{K_{PAS}}{(S)} \right)$, that is, the intercept is the same as that without inhibitor

$$\text{Slope} = \frac{1}{k(P)} \left[\frac{K_{PA} \cdot K_{PAS}}{(S)} + \frac{K_{PA} \cdot K_{PAS}(I)}{(S)K_{PI}} \right] = \frac{K_{PA} \cdot K_{PAS}}{K(P)(S)} \left(1 + \frac{(I)}{K_{PI}} \right)$$

Experiments were carried out in which O_2 consumption was measured in the presence of various concentrations of AAD with and without inhibitor.

Free AAD, (a), was calculated by the method suggested by Stadie and Zapp (17) using the following equation:

$$(a) = (A) - [(PA) + (PAS)] = (A) - \left[1 + \frac{K_{PAS}}{(S)} \right] (PAS)$$

They assumed that the O_2 uptake was directly proportional to the (PAS). This was determined from the data of Negelein and Bromel (21). Using a pure enzyme saturated with coenzyme and substrate they determined O_2 uptake in pyrophosphate buffer, at pH 8.3 and 38°C. Assuming a molecular weight of 70,000 they estimated that 1 gm. mole enzyme of purity 1 would cause the uptake of 2000 gm. moles of O_2 per minute.

In our experiments (PAS) was determined from the following equation:

$$\begin{aligned} (PAS) &= \frac{\text{mm.}^3 O_2 \text{ absorbed/hr./cc. reaction mixture}}{60 \times 22400 \times 2000} \\ &= \frac{\text{mm.}^3 O_2 \text{ absorbed/hr./cc. reaction mixture}}{2.688 \times 10^9} \end{aligned}$$

In the presence of inhibitor, assuming P combines with I giving PI , the free AAD would be determined in the same way as in the absence of inhibitor, namely by dividing the O_2 absorbed/hr./cc. in the presence of inhibitor by 2.688×10^9 . The value for K_{PAS} determined by Stadie and Zapp was used.

In several experiments we determined free AAD in the presence of inhibitor by the above method. In no case was the intercept in the presence of inhibitor

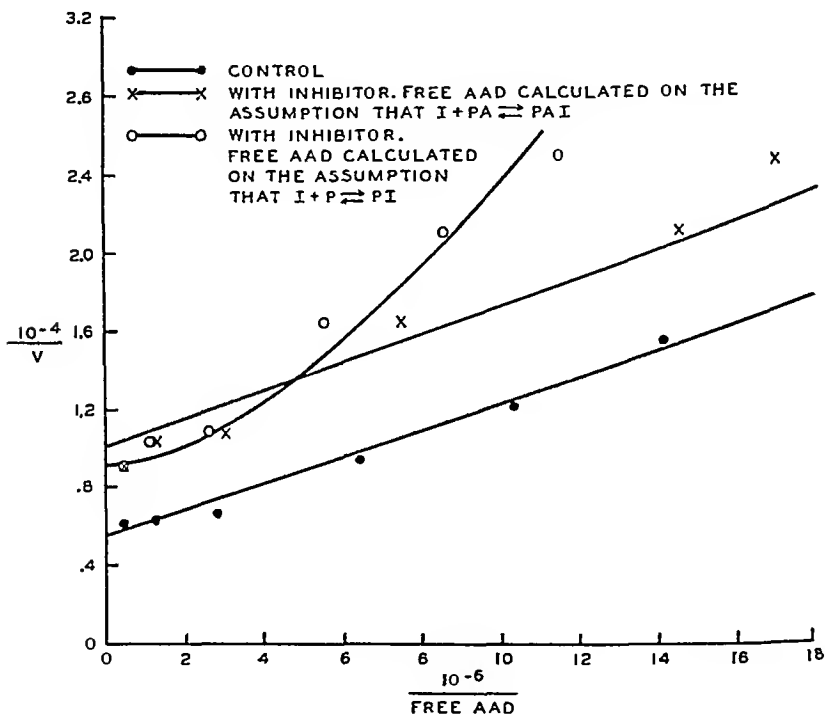
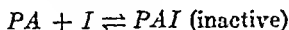
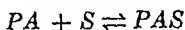
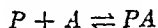


FIG. 2. EXPERIMENT 26. VELOCITY OF REACTION OF *d*-AMINO ACID OXIDASE WITH VARIOUS CONCENTRATIONS OF AAD

$\frac{1}{v}$ reciprocal moles O_2 /l./min.; $\frac{1}{\text{free AAD}}$ reciprocal moles uncombined AAD/l. ——— and x——x calculated "best" straight lines. Inhibitor: $Cu(OH)_2$ eluate, 0.14 mg. protein N/vessel.

equal to that in the absence of inhibitor so that I did not compete with AAD . Furthermore, when free AAD was determined on the assumption that all AAD was free which was not PA or PAS the curves for $\frac{1}{v}$ against $\frac{1}{(a)}$ were not straight but were parabolic (fig. 2) indicating that the calculated concentrations of free AAD were too high. This also pointed to the improbability of I combining with P to the exclusion of A .

2. The inhibitor combines with the enzyme-coenzyme complex.



$$(P) = (p) + (PA) + (PAS) + (PAI) \quad (1)$$

$$(A) = (a) + (PA) + (PAS) + (PAI) \quad (2)$$

$$K_{PA} = \frac{(p)(a)}{(PA)} \quad (3)$$

$$K_{PAS} = \frac{(PA)(S)}{(PAS)} \quad (4)$$

$$K_{PAI} = \frac{(PA)(I)}{(PAI)} \quad (5)$$

Combining equations 1, 3, 4 and 5,

$$\begin{aligned} (P) &= \frac{K_{PA}(PA)}{(a)} + \frac{K_{PAS}(PAS)}{(S)} + (PAS) + \frac{(PA)(I)}{K_{PAI}} \\ &= (PAS) \left[\frac{K_{PA} \cdot K_{PAS}}{(a)(S)} + \frac{K_{PAS}}{(S)} + 1 + \frac{K_{PAS}(I)}{(S)K_{PAI}} \right] \end{aligned}$$

If $v = k(PAS)$,

$$\frac{1}{v} = \frac{1}{k(P)} \left[1 + \frac{K_{PAS}}{(S)} + \frac{K_{PA} \cdot K_{PAS}}{(a)(S)} + \frac{K_{PAS}(I)}{(S)K_{PAI}} \right]$$

$$\text{Intercept} = \frac{1}{k(P)} \left[1 + \frac{K_{PAS}}{(S)} \left(1 + \frac{(I)}{K_{PAI}} \right) \right]$$

$$\text{Slope} = \frac{1}{k(P)} \left(\frac{K_{PA} \cdot K_{PAS}}{(S)} \right) \text{ that is, it is the same as that without inhibitor.}$$

In this case, since it is assumed that PA combines with I the free AAD could be determined from the following equation:

$$(a) = (A) - \left[1 + \frac{K_{PAS}}{(S)} \right] (PAS) - (PAI)$$

The amount of AAD bound as PAI was found by assuming that (PAI) is proportional to the difference in O_2 uptake in the presence and absence of inhibitor. This value ($mm.^3 O_2$ difference between the experiment without inhibitor and that with inhibitor) can be converted to (PAI) by dividing it by 2.688×10^9 as in the case for (PAS) . This value is then subtracted from (a) found in the absence of inhibitor. Table 6 gives the results in detail of a typical experiment.

Figure 3 is a graph derived from the data of this experiment. Free AAD was calculated in the manner described in this section.

In this experiment the intercepts of the curves for experiments with and without inhibitor are 1.11×10^4 and 0.502×10^4 . The probability that the difference in these values is due to chance is <0.01 . The slopes of these curves are 0.266×10^{-3} and 0.29×10^{-3} respectively. The probability that the difference between these values is significant is <0.01 . These analyses indicate, therefore, that the curve for the experiment with inhibitor has the same slope but a greater intercept than that without inhibitor. That the intercepts of curves with and without inhibitor are different signifies that the inhibitor does not compete with AAD, and that the curves tend to be parallel indicates that the inhibitor combines with the apoenzyme-coenzyme complex.

TABLE 6
Protocol of Experiment 288

A	O ₂ CONSUMPTION	PAS	α	$\frac{1}{\alpha}$	$\frac{1}{\nu}$
Control					
moles/l.	moles/min./l.	moles/l.	moles/l.		
21.8×10^{-7}	1.90×10^{-4}	0.950×10^{-7}	20.175×10^{-7}	0.0496×10^7	0.526×10^4
4.36×10^{-7}	1.699×10^{-4}	0.849×10^{-7}	2.908×10^{-7}	0.343×10^7	0.592×10^4
2.91×10^{-7}	1.489×10^{-4}	0.745×10^{-7}	1.637×10^{-7}	0.61×10^7	0.670×10^4
2.18×10^{-7}	1.280×10^{-4}	0.64×10^{-7}	1.085×10^{-7}	0.922×10^7	0.780×10^4
With inhibitor					
21.8×10^{-7}	0.91×10^{-4}	0.495×10^{-7}	19.68×10^{-7}	0.0508×10^7	1.10×10^4
4.36×10^{-7}	0.758×10^{-4}	0.4685×10^{-7}	2.4395×10^{-7}	0.411×10^7	1.324×10^4
2.91×10^{-7}	0.833×10^{-4}	0.3275×10^{-7}	1.3095×10^{-7}	0.764×10^7	1.20×10^4
2.18×10^{-7}	0.67×10^{-4}	0.305×10^{-7}	0.780×10^{-7}	1.28×10^7	1.494×10^4
	CONTROL	WITH INHIBITOR	n	T	P
Calculated intercept....	0.502×10^4	1.11×10^4	4	60	<0.01
Calculated slope.....	0.29×10^{-3}	0.266×10^{-3}	4	18.5	<0.01

In table 7 the values for intercepts calculated for "best" straight lines are given for several experiments. It will be seen that the values of intercepts obtained with and without inhibitor are significantly different for all cases.

S. Competition between inhibitor and substrate for enzyme. Table 8 gives the results in detail of a typical experiment (Experiment 30) in which O₂ consumption was determined for various concentrations of substrate, with and without inhibitor. Figure 4 represents the curves obtained from these data. The intercepts of "best" curves for experiments with and without inhibitor are 1.11×10^4 and 0.502×10^4 and the probability that the difference between these values is due to chance is <0.01 . The slopes are 0.266×10^2 and 0.29×10^2 respectively. The probability that these values are significantly different is <0.01 , that is, the curves are probably parallel.

Figure 5 shows curves obtained from an experiment in which several concentrations of inhibitor were used.

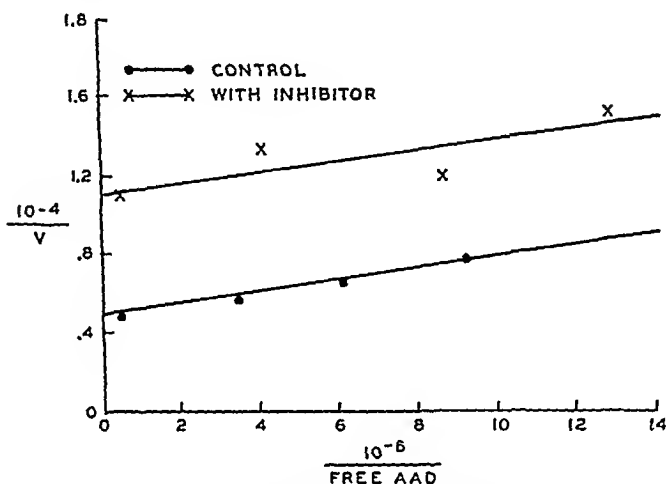


FIG. 3. EXPERIMENT 288. VELOCITY OF REACTION OF *D*-AMINO ACID OXIDASE WITH VARIOUS CONCENTRATIONS OF AAD

$\frac{1}{V}$ reciprocal moles O_2 /l /min ; $\frac{1}{\text{free AAD}}$ reciprocal moles uncombined AAD/l. Calculated "best" lines drawn. Inhibitor: ammonium sulphate precipitate, 0.135 mg. protein N/vessel.

TABLE 7

Significance of differences between intercepts of curves for experiments in the presence and absence of inhibitor when $\frac{1}{V}$ is plotted against $\frac{1}{\text{free AAD}}$

Intercept

EXPERIMENT	SUBSTRATE CONC. M	CONTROL	WITH INHIBITOR	INHIBITOR CONCENTRATION MG. PROTEIN N	n	t	P
284	0.0253	0.33 $\times 10^4$	0.70 $\times 10^4$	0.135	4	17.5	<0.01
292	0.0253	0.33 $\times 10^4$	0.50 $\times 10^4$	0.135	4	5.32	<0.01
294	0.0253	0.39 $\times 10^4$	0.459 $\times 10^4$	0.135	4	4.03	<0.02
16	0.0253	0.40 $\times 10^4$	0.85 $\times 10^4$	0.24	4	7.62	<0.01
2	0.006325	0.4615 $\times 10^4$	0.761 $\times 10^4$	0.135	4	32.6	<0.01
			0.716 $\times 10^4$	0.027	4	3.99	<0.02
288	0.006325	0.502 $\times 10^4$	1.11 $\times 10^4$	0.135	4	60.0	<0.01
			0.53 $\times 10^4$	0.0135	4	4.0	<0.02

The results of these experiments indicate that the inhibitor does not compete with the substrate.

Discussion. An increase in blood NPN is one of the changes known to occur in shock. This is partially attributable to an increase in free amino acids accord-

ing to Lurje (22) and Engel et al. (23). We have found that kidney samples from dogs in shock from hemorrhage show a considerable decrease in their ability to

TABLE 8
Protocol of Experiment 30

S d-ALANINE	$\frac{1}{S}$	CONTROL		WITH INHIBITOR	
		O ₂ CONSUMED	$\frac{1}{v}$	O ₂ CONSUMED	$\frac{1}{v}$
		moles/min /l		moles/min /l.	
0.0253	39.5	2.53×10^{-4}	0.395×10^4	1.085×10^{-4}	0.922×10^4
0.01265	79.1	2.352×10^{-4}	0.4251×10^4	0.982×10^{-4}	1.018×10^4
0.00843	118.6	2.202×10^{-4}	0.4540×10^4	0.96×10^{-4}	1.042×10^4
0.00562	178	2.152×10^{-4}	0.4645×10^4	0.2402×10^{-4}	1.352×10^4
0.0028	357	1.60×10^{-4}	0.6250×10^4		
0.0014	714	0.744×10^{-4}	1.3440×10^4	0.565×10^{-4}	1.77×10^4

	CONTROL	WITH INHIBITOR	n	T	P
Calculated intercept	0.273×10^4	0.9515×10^4	7	16.9	<0.01
Calculated slope	0.139×10^2	0.1197×10^2	7	4.41	<0.01

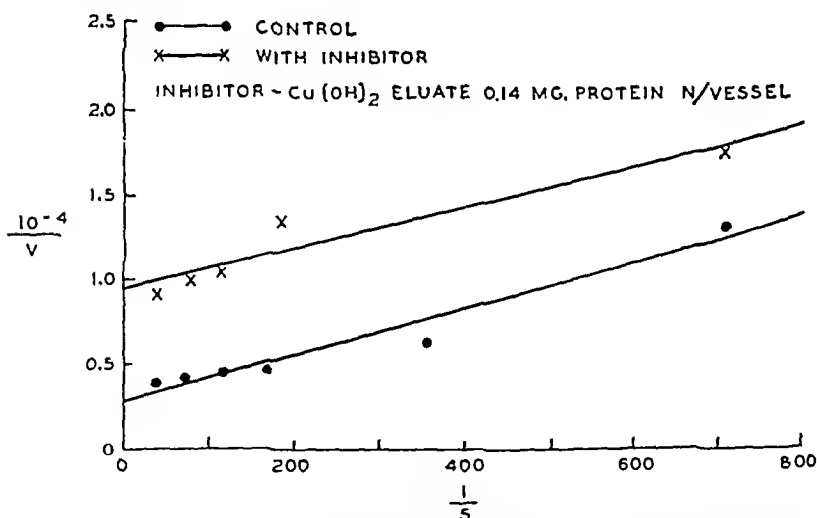


FIG. 4. EXPERIMENT 30 VELOCITY OF REACTION OF d-AMINO ACID OXIDASE WITH VARIOUS CONCENTRATIONS OF d(-)ALANINE

$\frac{1}{v}$ reciprocal moles O₂/l/min; $\frac{1}{S}$ reciprocal moles d(-)ALANINE/l. Calculated "best" lines drawn. Inhibitor $\text{Cu}(\text{OH})_2$ eluate, 0.14 mg protein N/vessel.

oxidise glutamic acid and alanine when compared with samples removed prior to bleeding (24). Such a decrease in metabolic ability could be due either to de-

struction of coenzyme or to destruction of the apoenzyme. It has been shown *in vivo* that shock will lead to an enzymatic destruction of the coenzymes, co-carboxylase, cozymase and AAD, and that anoxia produces destruction of co-carboxylase. In a preliminary communication we showed that anoxia decreased the ability of excised tissues to metabolise lactic and glutamic acids and alanine *in vitro* (11). The magnitude of the decrease was such that it could not be accounted for on the basis of coenzyme destruction alone. The assumption was made that during anoxia some factor, present in the tissues, comes into play which inhibits these enzyme systems in a manner apart from hydrolysis of the coenzymes. It was then found that tissue extracts did contain a thermolabile sub-

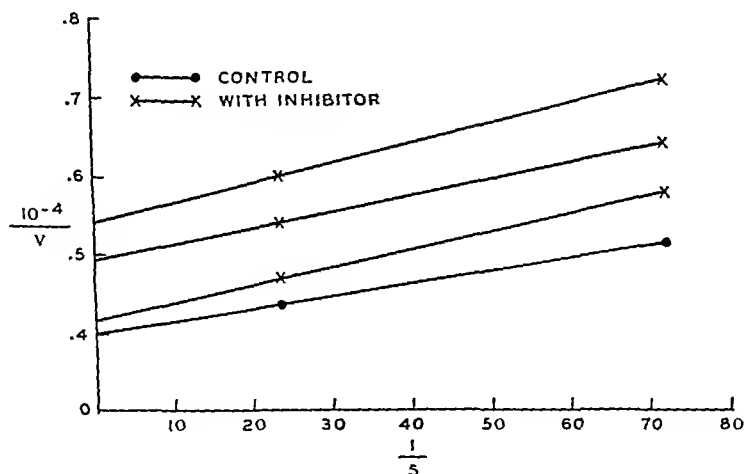


FIG 5 EXPERIMENT 227 VELOCITY OF REACTION OF *d*-AMINO ACID OXIDASE WITH VARIOUS CONCENTRATIONS OF *d*-ALANINE

$\frac{1}{V}$ reciprocal moles $O_2/l/min$; $\frac{1}{S}$ reciprocal moles *d*-alanine/l. Calculated "best" lines drawn. Inhibitor beef liver extract carried through ammonium sulphate precipitation. Highest concentration of inhibitor 0.4965 mg protein N/vessel; others diluted 1 and 5 and 1 in 25

stance which inhibits the oxidation of *d*-glutamic acid and *dl*-alanine by pigeon liver and also inhibits the isolated *d*-amino acid oxidase. The *d*-amino acid oxidase was chosen for further study because of its ease of preparation and because it has been obtained in a highly purified state. In addition, the kinetics of its reaction have been studied (13, 17). The inhibitor of this enzyme which we have isolated may or may not be similar to other enzyme inhibitors. The possibility of the liberation or activation of such inhibitors in shock, or the production by shock of conditions favorable for their action may be one explanation of the decreased metabolic activity which occurs in shock and in anoxia. Experiments are in progress to determine whether the injection of this inhibitor increases the blood amino acid content of dogs.

The factor is thermolabile, non dialysable and appears to be a globulin, since it can be precipitated by half saturation with ammonium sulphate, or saturation with sodium chloride or magnesium sulphate. It is resistant to freezing and can be stored indefinitely in solid CO_2 .

The activity varies with the pH and with the concentration of phosphate in the medium. At low pH's it exhibits its greatest inhibition. The low pH which exists in shock would, therefore, favor its activity. High pH's and high phosphate concentrations decrease the inhibition of *d*-amino acid oxidase by this factor.

It was reported in our preliminary paper that this inhibitor acted on the apoenzyme and did not affect the coenzyme. We have carried out kinetic studies in an attempt to determine the mechanism of inhibition. Experiments indicate that the inhibitor does not compete with the coenzyme for the apoenzyme, that is, it does not form an apoenzyme-inhibitor complex to the exclusion of the coenzyme. Neither does it compete with the substrate for the enzyme-coenzyme complex. Our experiments support the view that it combines to form an apoenzyme-coenzyme-substrate-inhibitor complex which is inactive. This complex is dissociable as has been shown by a decrease in inhibition produced by increasing the pH.

SUMMARY

The thermolabile, non dialysable inhibitor of *d*-amino acid oxidase has been further investigated.

It can be precipitated by half saturation with ammonium sulphate and by saturation with magnesium sulphate or sodium chloride.

Adsorption on and elution from cupric hydroxide increases the purity.

The solubility properties indicate that it is a globulin like substance.

Phosphate in high concentrations inhibits its activity.

The activity is greater at low pH's than at high pH's.

The combination with *d*-amino acid oxidase appears to be reversible since a decrease in inhibition is observed when the pH is raised.

Kinetic studies indicate that it does not compete with the coenzyme for the apoenzyme, neither does it compete with the substrate for the apoenzyme-coenzyme complex. It appears to form an apoenzyme-coenzyme-substrate-inhibitor complex.

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ANTITHYROID STUDIES. I. THE GOITROGENIC ACTIVITY OF SOME THIOUREAS, PYRIMIDINES AND MISCELLANEOUS COMPOUNDS

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It has been shown that certain derivatives of aniline and of thiourea inhibit thyroid hormone synthesis in the thyroid gland (1) (2). As a consequence of prolonged treatment with these agents, the thyroid becomes exhausted of its stores of thyroxin, blood concentration diminishes and a hypothyroid state supervenes (3). Following an experimental investigation in rats of a large number of compounds which on the bases of their chemical configuration might have antithyroid activity, Astwood (4) selected thiouracil as having the most promise from the point of view of potency and low toxicity. Subsequent clinical studies with thiouracil by Astwood and many others have shown that this compound effectively controls the manifestations of hyperthyroidism and that metabolic rates are reduced to normal levels. However, toxic reactions which are encountered would seem to limit general use of thiouracil in prolonged treatment of thyroid disease. In view of the above observations and the importance of such drugs in the treatment of thyrotoxicosis, a systematic study of several chemical types of compounds was undertaken in an effort to find drugs having greater activity than thiouracil and lesser toxicity within the therapeutic range.

Recent studies by Astwood and Bissell (5) and by Larson and his associates (6) confirm the earlier conclusions of Astwood that thiourea, thiouracil and related compounds act primarily by blocking the utilization of iodine and that thyroid hyperplasia occurs as a secondary compensatory response by the pituitary as a consequence of diminution of circulating thyroxin. The net result is a decrease in total thyroid iodine concentration which in the case of thiouracil, Astwood and Bissell have shown is proportional to the amount of goitrogenic agent administered. Since thyroid iodine concentration can be determined simply and with accuracy, we have used the iodine content as a means of estimating antithyroid activity of the compounds tested.

All of the more active compounds described by Astwood and others contain within their molecule the —NHCSNH— grouping and this structure has been retained for the most part in the compounds which have been tested in this study. However, certain liberties have been taken with this configuration, for example, one of the nitrogen atoms of certain heterocyclic ring compounds has been replaced by a sulfur atom. Also a group of aniline derivatives have been studied since Astwood has shown that they induce hyperplasia when given to rats in large doses. In addition, a group of miscellaneous compounds possessing an SH group alone or an analog of such compounds have been included in this search for active agents.

METHOD. The method suggested by Astwood and Bissell (5) was modified by using immature rats (26-28 days) weighing 40-45 grams at the beginning of the 10 day test period rather than 100 gm. rats. The rats in groups of three were placed in wire bottom metabolism cages and maintained in air-conditioned quarters at 25°C. The diet consisted of a standard breeder ration containing yellow cornmeal 33, whole wheat flour 31, whole milk powder 21, linseed meal 7, brewer's yeast (Anheuser Busch G) 3, alfalfa meal 2, dried hog liver 2, CaCO_3 0.5 and iodized NaCl 0.5 parts. Access to food was through a side arm feeder attached to the cage. Scattering of food was negligible.

Early in the course of these studies it was observed that thyroids of rats on the above diet contained less than half as much iodine as was found by Astwood and Bissell. In order to avoid any possibility of an inadequate iodine intake and to increase thyroid iodine concentration so that it was comparable to that of the Boston colony, KI was added to the drinking water. This, with the iodine of the iodized salt in the food, insured a daily intake of approximately 12 μg iodine over that in other constituents of the diet.

Compounds to be tested were mixed with the food except in the case of a few water soluble liquids which were dissolved in the drinking water. Food and water intakes and body weight were recorded every other day. The amount of compound which each rat received was calculated from the total food or water consumption over the ten day test period and is expressed as milligrams daily per kilogram of body weight. Average body weight over the test period was used for purposes of calculation.

The rats were killed with chloroform and the thyroids dissected free from adjacent tissue and capsule. Pooled glands from each group of 3 rats were weighed and analyzed for total iodine by Astwood and Bissell's modification of Kendall's method.

ESTIMATION OF ANTITHYROID ACTIVITY: Thiouracil was taken as a standard for comparison and activity values were assigned to compounds having antithyroid effects in terms of comparable doses which would reduce thyroid iodine concentrations to equivalent levels. For this purpose and for comparison of toxicity, thiouracil was administered to various groups of rats over a wide range of concentration in the food. The results are condensed in table 1 along with several groups of control rats on the diet without thiouracil.

When thyroid iodine concentration values from these data are plotted on ordinates against the logarithm of the dose of thiouracil on the abscissa as calculated from its concentration in the food and the average food intake, the points fall on a curve which seems to be best fitted by a straight line. This was true especially in the range of minimally effective doses to those which reduced iodine concentrations from the control level of 64 mg. per cent to 10 mg. per cent. Inspection of Astwood and Bissell's thiouracil curve shows that values of thyroid iodine concentration plotted against the logarithm of the concentration of the drug in the drinking water was also nearly linear in that part of the curve showing the most rapid change in iodine concentration with increasing doses. Admittedly, on theoretical grounds the dose response curve may be logistic in character, becoming asymptotic at 2 mg. per cent iodine concentration. However, to facilitate the evaluation of many compounds by screening tests it was assumed that the curve was linear within the limitations adopted. The straight line curve for thiouracil was calculated by the method of least squares and has been used as a standard (fig. 1).

Preliminary evaluation of all unknowns studied was done by giving daily doses of approximately 10 and 100 mg. per kilogram body weight in the food or water. Information obtained from such screening tests eliminated from further testing the relatively inactive compounds or those having greater toxicity than thiouracil. It may be observed from table 1 and figure 1 that maximal reduction in thyroid iodine concentration occurs at approximately 10 mg thiouracil per kg rat body weight daily (0.01% in food). The 100 mg. dose employed in screening tests not only served as a means of evaluation of goitrogenic activities of compounds less potent than thiouracil but yielded information as to general toxicity. It must be emphasized, however, that evidence of toxicity by these tests is limited entirely to data from body weight change and reduction in food or water intake. While it may be argued that failure to gain weight may have been due to decreased food or water intake and

was therefore an inadequate criterion of toxicity, inspection of the data revealed that in most cases decreased weight gains occurred at levels of drug intake at which little or no change in food consumption took place.

Compounds which caused reduction in thyroid iodine concentration below 10 mg. per cent during preliminary screen tests were retested at one or more lower concentrations in the diet. In a number of such compounds so tested sufficient data were secured so that a complete dose response curve could be plotted. Figure 2 shows linear curves for several of these compounds. The lines were calculated by the least squares method from experimental data. It was observed that in all but one of the ten compounds which have been plotted in this manner, angles of the curves on semi-logarithmic, 4 cycles $\times 10$ to the inch K & E graph paper, were almost identical, $128^\circ \pm 3.7^\circ$. It was assumed, therefore, that the log dose response was linear for all compounds, and that the lines obtained by plotting the data were parallel to each other.

TABLE 1
2-thiouracil

COMPOUND	FORMULA	NUMBER OF ANIMALS	CONCENTRATION IN FOOD	AVERAGE DOSE	BODY WT. GAIN	THYROID WT.	THYROID IODINE	ESTIMATED ACTIVITY THIOURACIL = 100
			per cent	mgm./kgm./day	gm./day	mgm./100 gm. rat	mgm. per cent	
Controls		50			$3.2 \pm .6$	$7.5 \pm .8$	63.6 ± 9.1	
2-thiouracil	$\begin{array}{c} \text{O}=\text{C}-\text{NH} \\ \quad \\ \text{HC} \quad \text{C}=\text{S} \\ \quad \\ \text{HC}-\text{NH} \end{array}$	3	0.00015	0.2	3.4	7.1	53.9	100
		9	0.0003	0.36	3.5	6.9	39.9	
		12	0.0006	0.4	3.3	7.4	38.7	
		12	0.0013	1.2	3.7	6.7	34.6	
		6	0.003	3.5	3.3	8.8	18.1	
		6	0.005	5.3	3.0	10.1	15.0	
		6	0.0065	7.5	3.4	10.6	7.2	
		6	0.01	10.6	3.1	14.6	5.1	
		9	0.05	52.0	3.0	26.2	3.2	
		9	0.10	103	3.0	32.6	1.4	
		3	0.30	190	1.9	28.1	0.8	
		3	0.60	578	1.4	35.8	0.8	
		6	1.0	756	1.0	30.3	2.0	

The level of 12 mg. per cent thyroid iodine concentration was selected as the base line for comparing the activity of unknown agents and thiouracil. This base was chosen for several reasons. It represented a definite response to active antithyroid compounds well beyond any animal variability or analytical error in iodine determination and at the same time remained within the unquestionably linear portion of the dose response curve. Also, as Astwood (7) has pointed out, it is near this level of iodine concentration that increases in thyroid weight occur in the 10 day test period.

Relative activity of compounds shown in the tables was calculated by first plotting thyroid iodine concentration values against dose on 4-cycle semi-logarithmic paper. Provided these points lay within the range of 10-40 mg. per cent iodine, one or more of them were projected to the 12 mg. per cent base line along the 128° slope line. The intercept on the 12 mg. per cent line gave the dose of compound which theoretically reduced thyroid iodine to this level. The ratio of this dose or an average of several of such doses, to the corresponding

intake (6.4 mg. per kg.) of thiouracil which reduces thyroid iodine to 12 mg. per cent (fig. 1) is the estimated relative activity. When sufficient data was available (fig. 2) to plot points through which a dose response curve could be drawn, the dose corresponding to the intercepts of these lines with the 12 mg. per cent base was used to calculate relative activity.

RESULTS. THIOUREAS AND PYRIMIDINES: Four compounds, thiourea, *sym*-diethylthiourea, *sym*-dibutylthiourea and phenylthiourea which have

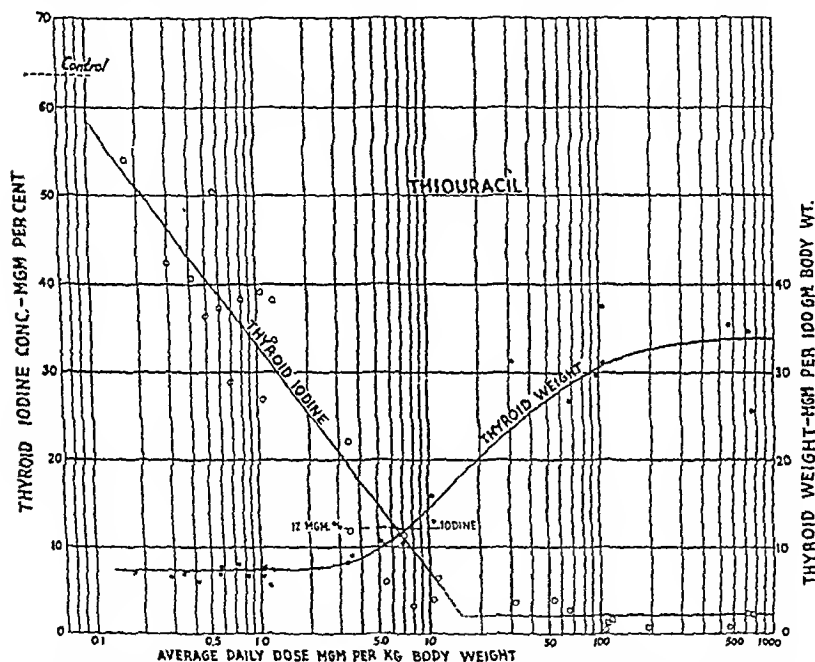


FIG. 1. RELATIONSHIP OF DOSE OF THIOURACIL TO TOTAL THYROID IODINE CONCENTRATION AND THYROID WEIGHT OF RATS AFTER ADMINISTRATION FOR TEN DAYS

Thiouracil was given in the food and the dose shown on the abscissa (plotted on logarithmic scale) was calculated from concentration in the food and average daily food intake. The thyroid iodine curve was fitted to the experimental data by the method of least squares. The equation for this line is $I = 32.8 - 25.9 \log D$ where I is iodine concentration in mg per cent and D is dose in mg per kg. body weight. The dose of thiouracil which according to the equation reduces thyroid iodine to 12 mg per cent is 6.4 mg. per kg. and was assigned a value of 100.

been previously investigated (1) (2), have also been tested in this study for purposes of comparison. In addition, eight other thiourea derivatives have been screened. In general they were all less active than thiouracil. Dibutylthiourea was much less active than diethylthiourea which in turn was more active than thiourea or ethylthiourea. The relative activity of these compounds is shown in table 2.

The pyrimidines and hexahydropyrimidines which have been studied are

substituted in the 2-position by sulfur except 2,4,6-trichloropyrimidine. Of the substituted thiouracil types, 4-methylthiouracil was slightly more effective than thiouracil while the *N*-phenyl derivative showed only slight activity and was also toxic (table 3).

It is interesting to note that reduction of thiouracil and substitution in the 1-position resulted in loss of activity as demonstrated by the data obtained for

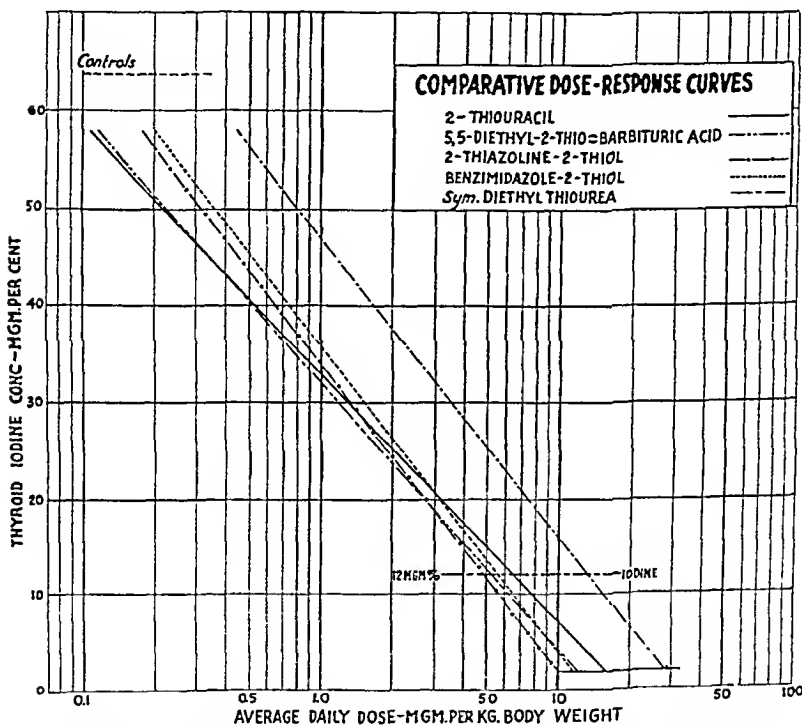


FIG. 2. CURVES SHOWING RELATIONSHIP BETWEEN THYROID IODINE CONCENTRATION AND DOSE OF THIOURACIL, 5,5-DIETHYL-2-THIOBARBITURIC ACID, 2-THIAZOLINE-2-THIOL, BENZIMIDAZOLE-2-THIOL AND SYM-DIETHYLTHIOUREA

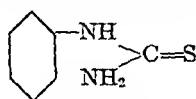
The compounds were given to rats in their food for a period of ten days. The average daily dose was calculated from the concentration in the food and the average food intake. The lines, calculated by the method of least squares from experimental data show the similarity of slopes. Activity was calculated from the dose under the intercept of the slope line and the 12 mg. per cent base line, thiouracil being assigned a value of 100.

1-butyl-2-thiohexahydropyrimidine. It can be seen from table 2 that whereas 2-thiobarbituric acid and its 5-nitroso and 5-methyl derivatives are inactive, some of the di-substituted derivatives of this 2-thiohexahydropyrimidine possess pronounced activity. Thus 5,5-diethyl-2-thiobarbituric acid is more active than thiouracil. However, increasing the length of the alkyl side-chains, resulted in decreased activity in this disubstituted series. The relative

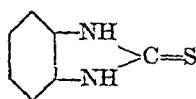
activity of thiobarbital and thiouracil varies apparently at different dosage ranges. According to Astwood, Bissell and Hughes (8), thiobarbital is more active than thiouracil at the lower dose ranges (0.003–0.02 per cent in food) but less active at the higher (0.02–0.1 per cent). While our data do not lend themselves to accurate activity comparisons at different intakes of drugs, it is evident that the dose of thiobarbital that reduces the thyroid iodine concentration to 12 mg. per cent is less than that of thiouracil. We found, however, in contrast to the observations of Astwood and coworkers, that thiobarbital was more toxic than thiouracil at all levels of drug intake. Thus 88 thiouracil fed rats showed an average weight gain of 3.3 grams per day during the ten day test period whereas 24 rats given thiobarbital in a similar dose range gained only 2.7 grams per day.

MISCELLANEOUS COMPOUNDS: During the course of investigating the anti-thyroid activity of sulfur-containing compounds, a number of miscellaneous compounds available in our laboratories were fed to rats in the routine manner. These compounds and their relative potencies are listed in table 4. Pyridine-2-thiol, thiazoline-2-thiol and benzimidazole-2-thiol were the most active compounds found in this miscellaneous series. Derivatives of these substances are being investigated and will be the subject of another report. The straight line curves for mercapto-2-thiazoline and benzimidazole-2-thiol are shown in figure 2.

It is interesting to note that when phenylthiourea having an activity of 14, is compared with benzimidazole-2-thiol with an activity of 116, ring closure has resulted in greatly enhanced activity:

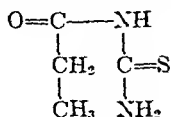


Phenylthiourea
(14)

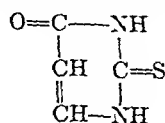


Benzimidazole-2-thiol (keto form)
(116)

This increased effect may be due to the fact that the benzimidazole-2-thiol is a stronger acid than phenylthiourea and exists predominately in the enol form. Whether the acidity or ease of reduction of these compounds can be correlated with activity remains to be established. Another such comparison, although not strictly analogous, is that of propionyl thiourea and thiouracil:



Propionylthiourea
(14)



Thiouracil
(100)

Strict comparison should be made with 4,5-dihydrothiouracil, which Astwood (6) has found to be only ten per cent as active as thiouracil, and therefore this

TABLE 2
Thioureas

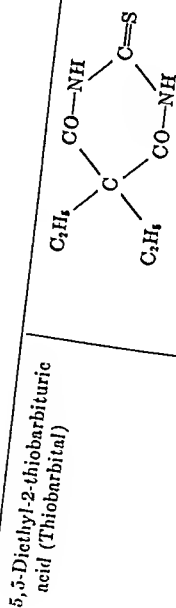
COMPOUND	FORMULA	NUMBER OF ANIMALS	CONCENTRATION IN FOOD	AVERAGE DOSE	BODY WT. GAIN	THYROID WT.	THYROID IODINE	ESTIMATED ACTIVITY THIOURACIL ≈ 100
			per cent	mgm./ kgm./ day	gm./day	mg./100 gm. rat	mgm. per cent	
Thiourea	$\begin{array}{c} \text{NH}_2\text{CNH}_2 \\ \parallel \\ \text{S} \end{array}$	3	0.01	12	3.6	8.8	36.7	9
		3	0.05	58	3.4	18.0	16.2	
		3	0.10	108	2.6	34.4	2.7	
		3	0.50	420	1.5	26.3	2.3	
Ethyl thiourea	$\text{C}_2\text{H}_5\text{NHCNSH}_2$	3	0.001	1.3	3.9	6.0	42.8	35
		3	0.01	12.0	3.3	10.5	17.4	
		3	0.10	105	2.1	20.1	3.1	
Diethylthiourea	$\begin{array}{c} \text{C}_2\text{H}_5\text{NHCNHC}_2\text{H}_5 \\ \parallel \\ \text{S} \end{array}$	3	0.001	1.2	3.8	5.9	43.0	47
		3	0.003	3.7	4.0	3.7	32.8	
		3	0.006	7.4	3.9	7.4	17.8	
		3	0.01	13	4.3	9.8	12.6	
		3	0.10	93	2.7	24.8	6.2	
Dibutylthiourea	$\text{C}_4\text{H}_9\text{NHCNSHC}_4\text{H}_9$	3	0.01	11	2.9	7.3	56.4	9
		3	0.10	95	2.4	11.7	8.9	
Phenylthiourea	$\text{C}_6\text{H}_5\text{NHCNSH}_2$	6	0.01	11	1.9	8.3	31.7	14
		6*	0.10	62	-4.0	21.2	8.5	
N-(4-Morpholinemethyl)thiourea	$\text{O}(\text{CH}_2\text{CH}_2)_3\text{N}-\text{CH}_2\text{NHCNSH}_2$	3	0.01	11	3.3	7.6	59.9	3
		3	0.10	111	2.6	10.9	20.3	
s-Di-(α -methylbenzyl)thiourea	$\text{C}_6\text{H}_5\text{CH}(\text{CH}_3)\text{NHCNSHCH}(\text{CH}_3)\text{C}_6\text{H}_5$	3	0.01	11	2.9	7.3	62.4	0
		3	0.10	102	2.6	6.8	44.6	

s-Di-(p-tert-amylphenyl)thiourea	$l\text{-C}_6\text{H}_4\text{C}_6\text{H}_4\text{NHCSNHC}_6\text{H}_4\text{C}_6\text{H}_4\text{H}_4\text{C}$	3	0.01 0.10	12 105	3.6 3.1	5.9 6.2	55.3 70.3	0
N-Propyl-N'-diphenylacetylthiourea	$\text{C}_6\text{H}_5\text{NHCSNHCOCCH}(\text{C}_6\text{H}_5)_2$	3 3	0.01 0.10	12 98	2.1 0.4	6.9 7.1	46.2 50.2	0
N-iso-Butyl-N'-diphenylacetylthiourea	$\text{C}_4\text{H}_9\text{NHCSNHCOCCH}(\text{C}_6\text{H}_5)_2$	3 3	0.01 0.10	12 98	3.1 1.0	6.0 6.1	40.0 48.1	0
Propionylthiourea	$\text{C}_3\text{H}_7\text{CONHCSNH}_2$	3 3	0.01 0.10	11 90	1.9 1.9	6.4 19.5	49.4 2.7	14
S-Carboxymethylisothiouron hydrochloride	$\text{NH}_2(\text{NH}=\text{C})\text{S}-\text{CH}_2\text{COOH}\cdot\text{HCl}$	3 3	0.01 0.10	11 113	2.9 3.4	6.6 6.3	56.5 48.9	0
S- α -Naphthylmethylisothiouron hydrochloride	$\text{NH}_2(\text{NH}=\text{C})\text{S}-\text{CH}_2\text{C}_{10}\text{H}_7\cdot\text{HCl}$	3 3	0.01 0.10	12 131	3.1 3.9	6.6 7.1	67.4 37.5	1

* One rat died on fifth day of treatment.

TABLE 3
Pyrimidines and thiobarbituric acids

COMPOUND	FORMULA	NUMBER OF ANIMALS	CONCENTRATION OF FOOD	AVERAGE DOSE	BODY WT. GAIN	THYROID WT.	THYROID IODINE	ESTIMATED ACTIVITY THIOURACIL = 100
			per cent	mgm./kgm./day	gm./day	mg./100 gm. rat	mgm. per cent	
2, 4, 6-Trichloropyrimidine	$\text{ClC}=\text{CHC}(\text{Cl})=\text{NC}(\text{Cl})=\text{N}$	3	0.01	10	2.8	7.1	63.6	0
		3	0.10	104	1.9	8.7	77.2	
4-Methyl-6-hydroxy-2-mercapto- pyrimidino (4-methyl- thiouracil)	$\text{HOC}=\text{CHC}(\text{CH}_3)=\text{N}-\text{C}(\text{SH})=\text{N}$	6	0.01	12	3.9	17.4	3.0	104
		6	0.10	105	2.6	30.6	1.9	
N-Phenylthiomethyluracil	$\text{CH}_3\text{C}=\text{CHCON}(\text{C}_6\text{H}_5)\text{CSNH} (?)$	3	0.01	11	1.8	5.5	46.7	3
		3	0.10	106	2.1	8.3	21.0	
1-Butyl-2-thio-hexahydro- pyrimidine	$\text{CH}_3\text{CH}_2\text{CH}_2\text{N}(\text{C}_4\text{H}_9)\text{CSNH}$	3	0.01	11	2.4	8.5	57.6	7
		3	0.10	96	2.2	11.3	12.1	
2-Thiobarbituric acid	$\text{COCH}_2\text{CONHCNSH}$	3	0.01	11	2.7	7.4	47.6	0
		3	0.10	113	2.9	7.4	48.8	
5-Nitroso-2-thiobarbituric acid	$\text{COCH}(\text{NO})\text{CONHCNSH}$	3	0.01	12	3.4	8.1	55.8	0
		3	0.10	113	3.6	5.8	50.3	
5-Methyl-2-thiobarbituric acid	$\text{COCH}(\text{CH}_3)\text{CONHCNSH}$	3	0.01	11	2.9	7.7	68.9	0
		3	0.10	115	3.3	6.9	62.4	



5-Ethyl-5-isoamyl-2-thio-barbituric acid

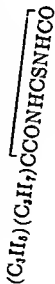
5-Ethyl-5-n-hexyl-2-thio-barbituric acid

5-Ethyl-5-phenethyl-2-thio-barbituric acid

5-Allyl-5-iso-propyl-2-thio-barbituric acid

5-Allyl-5-sec-butyl-2-thio-barbituric acid

5-Allyl-5-(1-methylbutyl)-2-thiobarbituric acid



3	0.0006	0.6	2.2	7.1	39.4	123
3	0.001	1.2	2.9	6.9	33.1	
3	0.003	3.1	2.7	7.9	17.9	
3	0.006	6.3	2.8	14.5	5.9	
3	0.01	10	2.7	17.7	4.7	
3	0.03	33	3.0	18.1		
3	0.06	63	2.6	21.9	1.5	
3	0.10	108	2.9	20.5	2.1	
3	0.3	300	2.5	23.5	1.9	
3	0.01	12	3.6	7.0	37.3	5
3	0.10	105	2.0	10.8	23.1	
3	0.01	12	3.6	7.9	23.7	0
3	0.10	105	2.6	9.5	16.1	
3	0.01	11	2.8	7.5	47.3	0
3	0.10	115	3.7	6.5	42.4	
3	0.01	11	3.1	8.9	27.0	19
3	0.10	102	2.6	17.1	7.7	
3	0.01	13	3.9	6.9	21.0	25
3	0.10	100	2.8	13.6	13.1	
3	0.01	11	3.5	7.7	47.4	2
3	0.10	110	2.3	10.5	29.1	

TABLE 4
Miscellaneous Compounds

COMPOUND	FORMULA	NUM- BER OF AN- IMALS	CONCEN- TRATION IN FOOD	AVER- AGE DOSE	BODY WT. GAIN	THY- ROID WEIGHT	THYROID IODINE	ESTIMATED ACTIVITY THYROID- CIT = 100
			per cent	mg./kg./ day	gm./day	mg./100 gm. rat	mg. per cent	
Tetrahydro-2-cyclohexyl-2-thio-s-triazone	$C_6H_{11}NCH_2NHCSNHCH_2$	3	0.01	11	2.7	9.3	41.4	10
		3	0.10	90	1.0	18.1	6.9	
2-(Dihydrothiazolyl) mercaptoacetic acid	$S-CH_2CH_2N=C-SCH_2COOH$	3	0.01	12	3.9	6.9	65.7	1
		3	0.10	120	3.4	8.2	38.5	
5-Amino-2-mercaptopurazole hydrochloride hydrate	$N-C(NH_2)=N-C(SH)=N \cdot HCl \cdot H_2O$	3	0.01	11	3.4	7.6	50.8	7
		3	0.10	102	2.7	10.6	11.2	
5-Methyl-5-ethyl-2-thio-4-keto-oxazolidine	$(CH_3)(C_2H_5)CCONHCS-O$	3	0.01	11	3.2	8.1	43.0	4
		3	0.10	120	3.1	12.1	17.0	
Pyridine-2-thiol	$2-C_5H_4NSH$	3	0.01	10	3.4	9.9	25.8	23
		3	0.03	29	2.7	18.4	3.0	
2-Mercaptonaphthaleno	$2-C_{10}H_7SH$	3	0.10	82	1.0	27.2	4.3	
		3	0.01	11	3.0	7.4	57.9	0
4,4'-Diaminodiphenyl-sulfoxide	$NH_2C_6H_4SOC_6H_4NH_2$	3	0.10	100	2.5	8.1	58.1	
		3	0.01	12	3.3	7.4	40.3	12
4,4'-Diaminobenzil	$NH_2C_6H_4COCOC_6H_4NH_2$	3	0.10	83	1.2	21.4	5.3	
		3	0.01	11	3.7	6.9	31.3	15
		3	0.10	58	0.4	17.5	8.3	

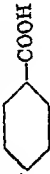
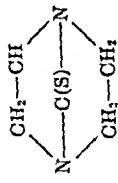
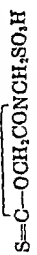
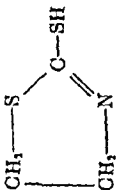
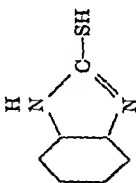
Sulfamide	$\text{NH}_2\text{SO}_2\text{NH}_2$	3	0.01	11	2.7	8.0	50.7	0
Piazthiol	$\text{C}_4\text{H}_4\text{N}_2\text{S}=\text{N}-\text{N}$	3	0.10	111	2.9	6.9	69.7	0
Triacetone amine	$(\text{CH}_3)_2\text{CCH}_2\text{COCH}_2\text{C}(\text{CH}_3)_2\text{NH}$	3	0.01	11	3.7	5.7	50.2	0
		3	0.10	110	3.7	5.4	43.5	
Thioammeline	$\text{NHCSNH}(\text{=NH})\text{NHC}=\text{NH}$	3	0.01	11	2.1	8.1	62.1	0
		3	0.10	100	1.8	8.5	45.5	
p-Aminobenzoic Acid		3	0.01	11	3.7	6.3	65.9	0.3
		3	0.10	110	3.8	5.5	70.7	
		3	1.0	1190	3.7	8.2	22.2	
		3	5.0	3980	0.8	23.5	4.9	
Ethyl p-aminobenzoate (Benzocaine)	$\text{NH}_2\text{C}_6\text{H}_4\text{COOC}_2\text{H}_5$	3	0.01	12	4.3	6.7	49.0	0
		3	0.10	118	3.9	7.4	63.6	
		3	1.00	1035	2.5	8.7	35.2	
p-Nitrobenzoic acid	$\text{NO}_2\text{C}_6\text{H}_4\text{COOH}$	3	0.01	11	3.3	6.1	71.8	tr.
		3	0.10	108	3.2	8.1	82.0	
		3	1.00	1040	1.8	9.8	29.5	
o-Nitrobenzoic acid	$\text{NO}_2\text{C}_6\text{H}_4\text{COOH}$	3	0.01	12	3.2	6.4	65.9	0
		3	0.10	99	2.9	6.8	82.1	
p-Mercaptobenzoic acid	$\text{p-HSC}_6\text{H}_4\text{COOH}$	3	0.01	13	4.0	6.2	59.7	0
		3	0.10	119	3.5	6.1	51.5	

TABLE 4—Concluded

COMPOUND	FORMULA	NUM- BER OF ANTI- MALS	CON- CENTRA- TION IN FOOD	AVER- AGE DOSE	BODY WT. GAIN	THY- ROID WEIGHT	THYROID IODINE	ESTIMATED ACTIVITY THYROID- CIT = 100
			per cent	mg./kg./ day	gm./day	mg./100 gm. rat	mg. per cent	
o-Mercaptobenzoic acid	o-HSC ₆ H ₄ COOH	3	0.01	11	3.1	6.2	40.9	tr.
		3	0.10	117	3.7	5.7	55.0	
		3	1.0	945	2.3	7.6	32.4	
		3	2.0	1500	-0.1	10.7	24.2	
p-Iodo-o-nitroaniline	p-I-O-NO ₂ C ₆ H ₃ NH ₂	3	0.01	12	3.2	6.5	49.5	0
		3	0.10	113	3.7	7.3	54.9	
p-Aminonacetanilide	p-NH ₂ C ₆ H ₄ NHCOCH ₃	3	0.01	12	3.6	5.7	59.3	0
		3	0.10	117	2.9	5.0	60.8	
p-Aminobenzyl-diethylamine ·2HCl	p-NH ₂ C ₆ H ₄ CH ₂ N(C ₂ H ₅) ₂ ·2HCl	3	0.01	12	3.7	5.7	71.8	0
		3	0.10	109	2.2	6.6	87.3	
3,7-Dimethyl-2,3-dihydro- 1,4-diazepine	$\overline{\text{CH}_2\text{C}=\text{CH}(\text{CH}_3)\text{C}=\text{N}-\text{CH}_2\text{CH}_2\text{N}}\text{H}$	3	0.01	12	3.6	6.5	67.2	0
		3	0.10	119	3.3	6.9	40.8	
bis(1-Amino-2-naphthyl) methane	C ₁₀ H ₆ (NH ₂)CH ₂ C ₁₀ H ₆ NH ₂	3	0.01	13	3.6	7.1	51.7	0
		3	0.10	126	3.6	6.3	56.0	
N-Phenylsulfonyl-N'-nico- tinyl hydrazine	C ₆ H ₅ SO ₂ NHNHCOC ₆ H ₄ N	3	0.01	11	2.9	6.8	41.2	0
		3	0.10	91	0.1	8.1	71.3	
Guanine hydrochloride di- hydrate	$\overline{\text{O}=\text{C}-\text{NHC}(\text{NH}_2)=\text{N}-\text{C}=\text{CNHCH}=\text{N}}\cdot\text{HCl}$ ·2H ₂ O	3	0.01	13	4.4	4.7	50.9	0
		3	0.10	114	3.3	5.5	53.0	

		3	0 01 0 10	12 104	2 9 2.8	6 3 6.5	30.8 38.8	0
1,4-Diazabicyclo [2,2,1] heptane-7-thione		3	0 01 0 10					
3-Sulfomethyl-2-thio-2,1 (3,5)-oxazolidione		3	0 01 0 10	13 119	1.1 3.8	6.5 6.0	49.0 34.1	11
2-Thiazoline-2-thiol (2-Mercaptothiazoline)		3	0.0003 0 006 0 001 0 003 0 005 0 006 0 01 0 03 0 06 0 10	0.3 0.6 1.1 3.0 5.5 5.8 10.0 30.0 54 78	3.3 3.2 3.3 3.4 2.8 3.3 3.4 3.4 2.0 1.7	5.9 7.5 7.6 8.1 13.9 14.1 25.2 24.6 30.2 31.2	46.4 39.6 36.3 19.4 7.2 4.4 2.8 2.1 1.4 2.9	131
Benzimidazole-2-thiol (2-Mercaptobenzimidazole)		6	0 0003 0 0006 0 001 0 003 0 006 0 01 0 03 0 055 0 10 0.50	0 3 0.7 1.1 3.3 6.4 11 28 50 79	3.2 2.8 3.1 3.0 3.3 3.2 2.5 2.3 1.4	6.5 7.3 8.7 10.7 11.1 23.1 33.8 39.2 43.6	51.3 39.4 36.1 14.9 13.0 3.6 1.6 1.9 3.7	116

* All rats dead on seventh day.

comparison does not follow the phenylthiourea-benzimidazole-2-thiol example. It is possible, however, that relationships similar to that of the phenylthiourea example will be found in the *alkyl*thiourea series and these are being investigated.

The results obtained with p-aminobenzoic acid do not agree entirely with Astwood's. Thyroid enlargement was slight and the dose response vs. decreased iodine was obtained only at the 1% and 5% levels. On the thiouracil activity scale p-aminobenzoic acid has 0.3% of the potency of thiouracil. Esterification of the carboxyl group (benzocaine) did not increase the activity nor did replacement of the amino group by the mercapto- or nitro-group. Astwood has shown that o-aminobenzoic acid is probably less active than the p-amino acid and the results in table 4 show no enhanced activity for the o-mercapto- nor o-nitrobenzoic acid.

SUMMARY

A group of 56 compounds have been tested for their ability to inhibit the function of the thyroid gland. The relative activity of these compounds has been determined by comparison with thiouracil using as a base the dose of the latter needed to reduce the thyroid iodine to 12 mg. per cent when the drug is administered to rats in the food for a ten day period. The relationship between the dose and the thyroid iodine concentration is a linear one for those compounds tested at several dose levels and it is postulated that other compounds will also give straight-lines having the same slope when the thyroid iodine concentration is plotted against dose. This assumption affords a practical means of determining the relative activities of comparable compounds.

The thioureas tested were less active than thiouracil. Similarly, the thiobarbituric acids investigated were also less active except 5,5-diethyl-2-thiobarbituric acid.

Of the miscellaneous compounds subjected to test, pyridine-2-thiol, thiazoline-2-thiol and benzimidazole-2-thiol were found to be the most active. The latter two have been tested at several dose levels and found to be more effective than thiouracil in the rat. Derivatives of these compounds and of pyridine-2-thiol are being investigated further.

Acknowledgment: We are indebted to Drs. E. B. Astwood of Harvard University and R. W. Rawson of Massachusetts General Hospital for many helpful suggestions during the course of this work, to the Misses Clare Blackford and Virginia Hinchman for their technical assistance and to the Research Chemists of the staff for many of the compounds studied.

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TOXICITY STUDIES ON BENZYL BENZOATE AND RELATED BENZYL COMPOUNDS

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Numerous clinical reports have appeared in the literature during the past decade in which the use of benzyl benzoate, isopropyl alcohol combinations have been highly recommended for the treatment of scabies. Kissmeyer (1) reported treating some 8000 cases of scabies in a three or four year period with remarkable success, and little or no serious post-therapeutic dermatitis. Goldman (2), Dauken (3), Ingles (4), Vellin (5) and others have reported similar clinical experiences employing benzyl benzoate solutions, lotions and ointments.

In determining the effect in small laboratory animals, of an acaricidal preparation containing benzyl benzoate 33% w/v, isopropanol 60% and cetyl alcohol, triethanolamine stearate and distilled water 20% w/v, it was found that cats are peculiarly hypersensitive to skin application. Preliminary experiments definitely showed that the isopropanol was not responsible for this toxic effect. These findings are in agreement with the extensive reports in the literature on the effects of isopropyl alcohol after oral administration or external application to the skin. Our attention was, therefore, directed to a study of the toxicity of benzyl benzoate and related benzyl compounds. Macht (6, 9, 12) Gruber (7, 8), Macht and Leach (10), Grant (11), Fuller and Hunter (13) Boruttau (14) and Baughton (15) have quite extensively studied the toxic and pharmacologic actions of these substances. This paper is concerned with further toxicity studies in which it was found that cats are peculiarly hypersensitive to skin applications of these benzyl compounds.

METHODS. *Acute oral toxicity:* The L.D.₅₀, orally by stomach tube, was determined in rats, rabbits, cats and dogs (table 1). All animals were starved twenty-four to forty-eight hours before administration. A total of 140 rats, 57 rabbits, 11 cats and 4 dogs were used for this part of the study. Each animal was used only once. After preliminary orientation tests, not less than three groups of five rats and three rabbits each were used in the range of $L.D_{50} \pm 0.5$ gram per kilo. All animals were observed for two weeks or until death following administration of the test substances.

Local application and absorption tests: The cats and dogs used for this part of the study had an area on their backs clipped with fine electric clippers. The clipped areas in the cats were approximately 4" by 6" or 7". The dogs had comparable areas clipped depending on the animals size. One of the dogs listed in table 3 had a much larger area clipped and received a greater quantity of the test solution.

The test solutions were applied to the backs of the animals by the use of cotton balls held firmly with haemostatic forceps. During the application the clipped areas were thoroughly massaged to facilitate absorption.

The test solutions applied to the larger domestic animals were applied with the use of a 4" pig bristle paint brush. Only the sheep in this group had its back clipped. The area clipped was comparable to that in the cats and dogs. Twenty cats, three dogs and

one horse, heifer, sheep and pig were used in this part of the study. All animals receiving these local applications were observed for a period of two weeks or until death.

RESULTS. The minimal lethal doses for the five compounds investigated are given in table 1. The L.D.50 in cats and dogs was determined for benzyl benzoate but not for benzyl alcohol, methyl and ethyl benzoate and benzyl acetate. As is evident from the table, dogs are much more resistant to benzyl benzoate than the other animals, since they tolerated approximately ten times the L.D.50 dose found for rats, rabbits, and cats without exhibiting toxic manifestations. All the compounds are of the same order of toxicity in rats and rabbits. Benzyl acetate was found to be somewhat less toxic than the others after oral administration. These results are about the same as Macht (6) obtained following subcutaneous and intraperitoneal administration, except in the case of the acetate. He found it to be somewhat more toxic than the others and attributed his results to faster absorbability.

TABLE 1
Acute oral toxicity of benzyl compounds in laboratory animals

COMPOUND	LD 50 IN GRAMS PER KILO			
	Rats	Rabbits	Cats	Dogs
Benzyl benzoate	2.8 (20)*	1.68 (12)	2.24 (11)	>22.44 (4)
Benzyl alcohol	2.08 (20)	1.04 (9)		
Ethyl benzoate	2.10 (45)	2.63 (12)		
Methyl benzoate	2.17 (25)	2.17 (12)		
Benzyl acetate	3.69 (30)	2.64 (12)		

* The number of animals used to obtain the L D 50 in each species is given in the parenthesis.

Toxic doses of the substances studied produced symptoms referable to central nervous system stimulation in rats, rabbits and cats. In general, the rats exhibited piloerection, muscular incoordination, progressive paralysis of the hind limbs and finally violent spastic convulsions, dyspnea and death, usually immediately preceded by respiratory paralysis.

In the rat and rabbit toxic doses of benzyl alcohol produced complete prostration within one-half hour following administration, and the animals remained in that condition until death. Rabbits receiving toxic doses of the other four substances usually exhibited central nervous system stimulation similar to that observed in the rat followed by a twelve to twenty-four hour period of prostration before death.

Cats which received toxic doses of benzyl benzoate exhibited effects similar to those observed in the rat and rabbit. Shortly after administration the cat developed excessive salivation, followed in two or three hours by generalized tremor, muscular incoordination, paralysis of the hind limbs, violent convulsive seizures and then prostration. Death usually occurred during the first twenty-four hours following administration. Respiratory paralysis usually immediately

preceded death. Cats appear to be able to tolerate benzyl benzoate orally about as well as rats and rabbits.

Three, twenty-four hour urine samples were collected from one of the cats and one of the dogs receiving benzyl benzoate orally. These samples were treated according to Roaf's method for the qualitative determination of hippuric acid as outlined in Hawk and Bergeim (16). A small quantity of white crystals were isolated from both of the first twenty-four hour samples. After recrystallization, the pure white substances melted at 187°C. Mixed melting points

TABLE 2

SUBSTANCE APPLIED	NO. OF CATS USED	AVG. NO. OF APPLICATIONS	APPROX. AMT. APPLIED EACH APPLICATION	RESULTS	AVERAGE SURVIVAL TIME
			cc.		hours
Isopropanol 60% Benzyl benzoate 33%	2	2	20	2 cats died	43
Ethyl alcohol 60% Benzyl benzoate 33%	2	2	20	2 cats died	46
Water 60% Benzyl benzoate 33%	2	3	20	2 cats died	69
Benzyl benzoate 100%	2	1	20	2 cats died	22
Benzyl benzoate 100%	2	2	7.5	2 cats died	48
Benzyl alcohol 100%	2	1	20	2 cats died	22
Benzoic acid* Isopropanol 60%	2	1	20	Cats survived. Apparently no absorption.	
Ethyl benzoate 100%	2	1	20	2 cats died	20
Methyl benzoate 100%	2	1	20	2 cats died	21
Benzyl acetate 100%	2	1	20	2 cats died	11

* 25% sol. of benzoic acid in 60% isopropanol.

taken with a known pure sample of hippuric acid were not depressed. No crystalline material could be isolated from any of the second and third twenty-four hour urine samples. These urine studies seem to indicate that the cat is capable of converting benzyl benzoate to benzoic acid which combines with glycine to form hippuric acid. It has been reported by Quick (17) that about 75 per cent of benzoic acid fed to a dog is excreted as glycuronic acid monobenzoate. Although these urine studies were only qualitative in nature, it is possible in the cat as in the dog, a large quantity of the benzyl benzoate administered was excreted as glycuronate.

From these acute oral toxicity studies it is apparent that the dog is peculiarly resistant to benzyl benzoate since he can tolerate more than ten times the quantity which is lethal to rats, rabbits and cats.

IRRITATION AND ABSORPTION STUDIES LOCAL APPLICATION IN CATS. The cats used for this part of the study were prepared as explained in the methods. The amount of each test solution applied was, of course, only approximate although an extreme effort was made to keep the amounts constant in each case.

Benzyl benzoate alone and in various vehicles, benzyl alcohol, ethyl and methyl benzoate, and benzyl acetate are all lethal to cats when applied locally in the amounts indicated in table 2.

A 25 per cent solution of benzoic acid in isopropanol 60% was nontoxic in the amounts applied, but this may be due to the fact that little or no absorption took place, as was evidenced by the white powder remaining on the cats' back after the test solution had dried.

TABLE 3

SPECIES	NO OF ANI- MALS	SUBSTANCE APPLIED	NO OF APPLICA- TIONS	APPROX AMT APPLIED EACH AP- PLICATION	RESULTS
				cc.	
Dog	2	Benzyl benzoate	6	100	No toxic symptoms
Dog	1	Benzyl benzoate	6	200	No toxic symptoms
Horse	1	Benzyl benzoate	5	1000	No toxic symptoms
Heifer	1	Benzyl benzoate	5	500	No toxic symptoms
Sheep	1	Benzyl benzoate	5	250	No toxic symptoms
Pig	1	Benzyl benzoate	5	200	No toxic symptoms

All the cats in this study that died did so in much the same manner. Generally, they first exhibited excessive salivation and twitching of the treated areas of their backs. This was followed somewhat later by generalized tremor, muscular incoordination, paralysis of the hind limbs and finally violent convulsions followed by respiratory failure and death. In some cases the cats remained completely prostrate following the convulsive seizures for many hours before death. In every case the cats that died lost from 200 to 400 grams in weight, undoubtedly caused by lack of intake of food and water.

LOCAL APPLICATION IN DOGS AND LARGER DOMESTIC ANIMALS. The dogs used for this part of the study were prepared in a manner similar to the cats receiving local applications. The horse, heifer and pig did not have the hair clipped.

Benzyl benzoate applied locally to dogs and larger domestic animals in the amounts indicated in table 3 were nontoxic. In all of the larger animals, the amounts of test solutions were only approximate, but were sufficient to thoroughly saturate their backs at each application.

SUMMARY AND CONCLUSIONS

1. The acute oral L.D.50 for benzyl benzoate, benzyl alcohol, methyl and ethyl benzoate and benzyl acetate was determined in rats and rabbits. The acute oral L.D.50 for benzyl benzoate only, was determined in cats and dogs.

2. All five compounds studied are of the same order of toxicity, orally in rats and rabbits. Cats are able to tolerate benzyl benzoate orally about equally as well as rats and rabbits. Dogs are considerably more resistant than any of the other three species to the oral administration of benzyl benzoate.

3. Hippuric acid was isolated in small amounts from the urine of both dogs and cats fed benzyl benzoate orally.

4. Benzyl benzoate alone and in various vehicles, benzyl alcohol, methyl and ethyl benzoate and benzyl acetate were all lethal when applied to the skin of cats.

5. External application of benzyl benzoate to dogs, horses, heifers, sheep and pigs was found nontoxic even after five or six daily applications.

The authors wish to express their appreciation for the assistance given them by Mr. W. E. Spafford in handling all the animals used in these toxicity studies.

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THE DESTRUCTION BY TYROSINASE OF THE IRRITANT PRINCIPLES OF POISON IVY AND RELATED TOXICANTS

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Three major methods of treatment of poison ivy and allied dermatites involve immunological methods (antigen injections), anti-pruritic treatments and the use of topical agents which are supposed to destroy the irritant materials. Among the latter ferric chloride and potassium permanganate solutions have been widely used for the destruction of the toxicants by oxidation. The use of harsh oxidants can be justified on the basis of present knowledge of the chemical structures of the irritant compounds. Studies by Majima and co-workers (1), McNair (2), Hill et al. (3), Mason and Schwartz (4), and Keil and co-workers (5, 6) have resulted in the isolation and identification of certain of the skin irritants of poison ivy, poison oak, Japanese lac and other plants of the *Anacardiaceae*. These compounds have been shown to be fat-soluble substituted phenols and catechols characterized by a long unsaturated side-chain attached to the ring. The toxic behavior of these irritants on the skin has been ascribed to the presence of both hydroxyl groups and side-chain (7).

In view of the chemical structures of the various *Anacardiaceae* and related toxicants (see 6, 8 and fig. 1), one might expect that these molecules would be oxidized to quinones by phenol oxidases. Since these oxidases attack such widely different compounds as niono- and polyphenols, tyrosine (9), adrenalin and certain sex hormones (10), it would seem likely that the phenolic groups of the poison ivy and allied toxicants might be attacked by these enzymes with resultant loss of irritant properties (2). The possibility of enzymatic action on the toxicants of poison ivy was pointed out by McNair (2) who observed that poison oak sap darkened upon exposure to air with an accompanying loss of toxicity (see also 11 and 12). He attributed this effect to the presence of an oxidase (laccase) in the sap. Recently in a preliminary note Sizer and Prokesch (13) have suggested that mushroom tyrosinase can inactivate poison ivy extracts in vitro. The present investigation is concerned with extending these studies to a wide variety of poison ivy preparations as well as to pure compounds which like poison ivy produce dermatitis. This study is also concerned with the inactivation by tyrosinase of poison ivy compounds after they have been placed on the human skin.

EXPERIMENTAL METHODS. (1) *Materials used.* Toxicants.¹ The poison ivy preparations were all commercial ones which had been obtained from the green leaves of the plant

¹ Poison ivy extracts furnished through the courtesy of Lederle Laboratories, Inc., E. R. Squibb and Sons, Arlington Chemical Co., Sharp and Dohme, Inc., Mulford Colloid Laboratories, Wyeth, Inc. Poison oak extract: Pitman-Moore Co. Kiurushi, urushiol,

by extraction with an organic solvent such as acetone, ether, or alcohol. Some of the preparations were further purified by removal of fats, waxes and chlorophyll. Most of these extracts were supplied dissolved in solvent, while the oleoresins were in paste form. Other natural toxicants included extracts of poison oak, Japanese kiurushi (obtained from crude Japanese lac) and extracted cashew nut shell liquid.

The purified materials utilized included cardanol (distilled from cashew nut shell liquid, anacardol (from the cashew nut), anacardic acid (from the cashew nut), tetrahydro-anacardol (synthetic), tetrahydro-anacardic acid (synthetic), urushiol (from Japanese lac) and 3-geranyl catechol (synthetic, 14).

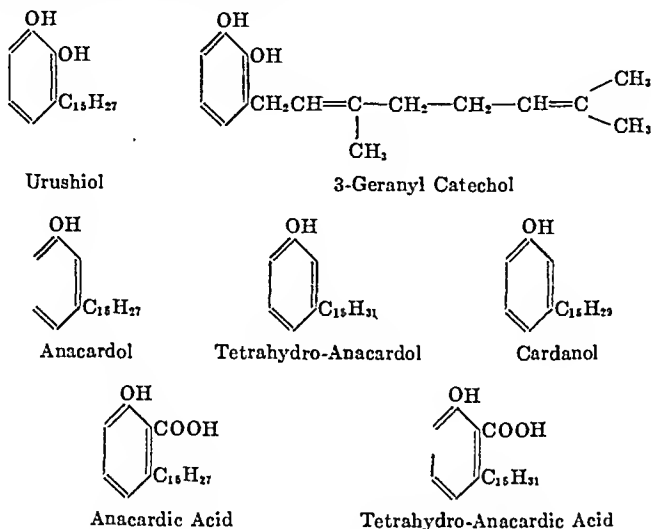


FIG. 1. STRUCTURAL FORMULAE OF POISON IVY AND RELATED TOXICANTS

*Tyrosinase preparations.*² These were prepared by Nelson from the edible mushroom, *Psalliota campestris*, according to the methods of Nelson et al. (9). Three different solutions were used which contained respectively 40,000, 6,000 and 4,200 Miller and Dawson catecholase units (15) and 500, 50 and 70 Adams and Nelson p-cresolase units (16).

(2) *Manometric studies.* The Barcroft differential respirometer was used at 37°C. at a shaking rate of 85 strokes per minute. Into the experimental flask were placed 1.0 ml. of poison ivy extract or 10% oleoresin solution (organic solvent subsequently removed by air drying),³ 0.1–0.3 ml. tyrosinase solution, and 0.1 M phosphate buffer of pH 7.3 to make a total volume of 3.3 ml. The control flask contained the same, except that buffer

3-geranyl catechol: Dr. Merrill W. Chase, Rockefeller Institute for Medical Research. Cashew nut oil preparations: Irvington Varnish and Insulator Co. Anacardic acid, anacardol, tetrahydroanacardic acid, tetrahydro-anacardol: T. E. Knock, Armour Research Foundation.

² We are greatly indebted to Dr. J. F. Nelson of Columbia University for the tyrosinase preparations.

³ Since the Mulford oleoresin was already suspended in buffer, the removal of the solvent was unnecessary.

or inactivated enzyme solution was substituted for the tyrosinase. The same technic was used with the purified toxic compounds, except that 0.02-0.05 ml. of substrate was utilized instead of 1.0 ml.

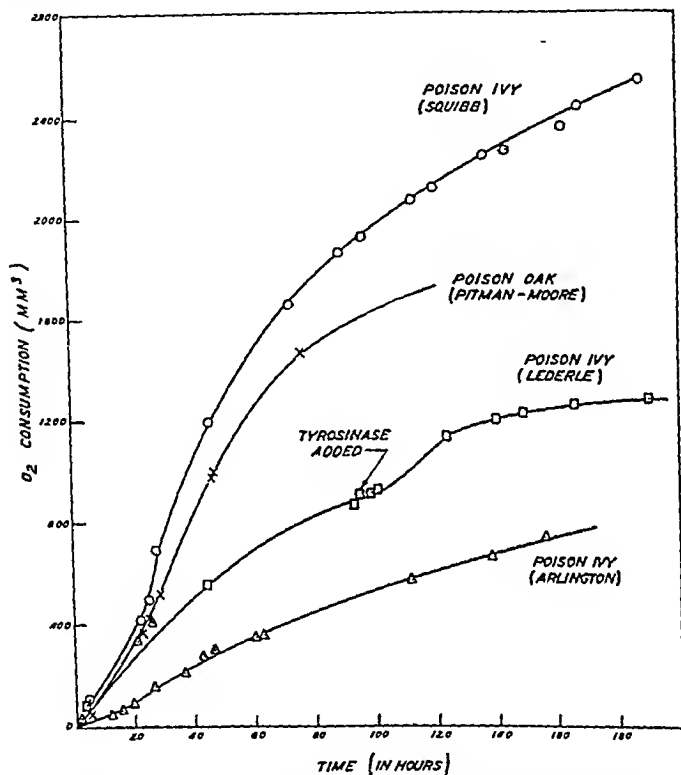


FIG. 2. THE OXIDATION BY TYROSINASE OF POISON IVY AND POISON OAK EXTRACTS

The experimental flask contained 0.3 ml. enzyme, 1.0 ml. extract (solvent evaporated off) and buffer to bring the volume to 3.3 ml. The control flask was the same but with buffer instead of enzyme.

The toxicants used were: Squibb poison ivy extract (2%); Pitman-Moore poison oak extract (5%); Lederle poison ivy extract (unprocessed), an additional 0.1 ml. tyrosinase was added after 96 hours; Arlington poison ivy (10%).

After adjustment to temperature for 5 to 15 minutes, the stopcocks were closed and manometric readings of oxygen consumption were taken at frequent intervals during the next 24 hours and then intermittently for one to five weeks. Since the manometers measure only a total of 500 mm.³ of gas, it was occasionally necessary to open the stopcocks for an instant so as to reequilibrate the height of the manometer fluid to zero.

RESULTS. Twenty-three experiments were performed with natural toxicants. In figure 2 are presented four typical results of experiments which have been

obtained with three different commercial extracts of poison ivy and one of poison oak. It may be seen from this figure that in some experiments a lag period of about ten hours is encountered before the reaction proceeds rapidly, while in other cases no lag period occurs. Although the reaction usually continues for more than 100 hours, the rate of oxygen consumption falls rapidly during the first days and then continues at a low value for a long period of time. In most reactions catalyzed by tyrosinase (9) the rate becomes retarded due to the inhibition of the enzyme by either substrate or reaction products or both. A similar inhibition of tyrosinase by poison ivy toxicants or their oxidation products seems to occur. If new enzyme is added after the rate has decreased (fig. 2), the reaction proceeds again at an accelerated pace. While in most cases the reaction becomes retarded due to inactivation of the enzyme, in a few studies, using only very small amounts of pure compounds, it appears likely that the substrate may have become exhausted (fig. 3). No significant difference in the kinetics of oxygen consumption was found among the three tyrosinase preparations used.

The action of tyrosinase on the oxidation of purified compounds was studied in 13 experiments, using cardanol, anacardol, anacardic acid, tetrahydro-anacardol, tetrahydro-anacardic acid, urushiol and 3-geranyl catechol. In all cases oxygen consumption curves were obtained which were similar to those shown in figure 3. Attention is called to the unusually long lag period in the case of anacardic acid. In general the results obtained with the purified compounds were strikingly similar to those with the crude toxicants, in spite of the fact that the former were much less miscible (and formed a separate phase) with the buffer than the latter.

Since many of the usual substrates of tyrosinase, such as catechol, pyrogallol, guaiacol, etc., undergo autoxidation with an accompanying darkening of color even in the absence of tyrosinase, the toxicants under consideration might be expected to undergo a similar reaction. In the usual manometric experiments using tyrosinase the results were independent of autoxidation, since the amount of toxicant was the same in both cups of the manometer. Autoxidation was investigated in 8 experiments by placing only buffer in the control cup and substrate plus buffer (without enzyme) in the experimental cup. The total volume in each cup was 3.3 ml. Typical results are presented in figure 4, from which it is apparent that both poison ivy extracts and purified toxicants undergo a gradual oxidation which is very much slower than when enzyme is present. In the case of the poison ivy extract (but not the purified compounds) the question arises as to whether or not the oxygen consumption is due to the presence of natural oxidases from the plant. This possibility is excluded, however, by experiments which indicate that autoxidation is not impeded by eliminating enzyme catalysis through heat inactivation (toxicant heated for 20 minutes at 90°C.) or by using an ether or acetone extract of the toxicant, since tyrosinase is insoluble in these solvents.

Since the reaction continued for a relatively long period of time in the absence of added disinfectant, there is a chance that the measured oxygen consumption

was actually due to bacterial contamination. This possibility is excluded by the following: 1) Since the toxicant was present in both flasks, bacterial growth was equally possible in both (although never observed in either), yet oxygen consumption was always greater in the experimental flask. 2) Many of the

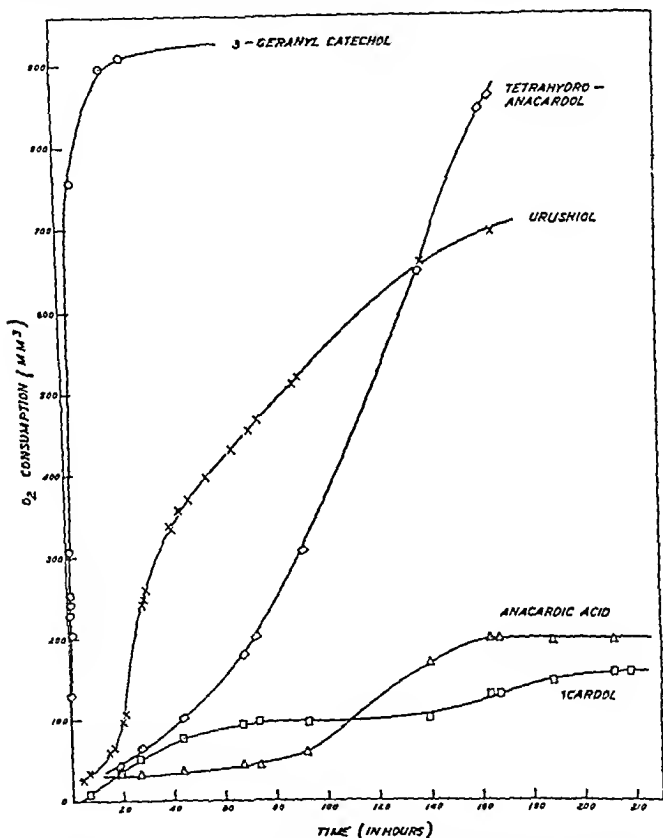


FIG. 3. THE OXIDATION BY TYROSINASE OF PURIFIED TOXICANTS

The control flask contained 3.3 ml. buffer, while the experimental flask contained 0.3 ml. enzyme, 3.0 ml. buffer, and one of the following: 0.05 ml. 3-geranyl catechol; 0.50 ml. tetrahydro-anacardol; 0.01 ml. urushiol; 0.05 ml. anacardic acid; 0.05 ml. anacardol.

toxicants are in themselves disinfectants. 3) In several experiments the anti-bacterial substance, toluene or sodium ethylmercuric thiosalicylate, was added to the digest, but the results on oxygen consumption were the same as usual.

(3) *Animal experiments.* Although the preceding manometric studies clearly indicate that mushroom tyrosinase catalyzes the oxidation of poison ivy toxicants and related irritant compounds, there is no indication from these experiments that the toxicant properties of these materials have been altered. In

order to obtain information on this problem, experiments were performed to determine the effect of tyrosinase on the toxicity of these compounds, as determined by their ability to produce dermatitis on humans and guinea pigs.

Since in most experiments the suspension in the manometer flasks was not homogeneous, it was customary to extract the fat-soluble toxicants from the digest with 5 ml. of ether. This was done with both the experimental and control flasks. Other investigators (5, 17-20) have clearly shown that the patch test is the method of choice in demonstrating quantitative sensitivity

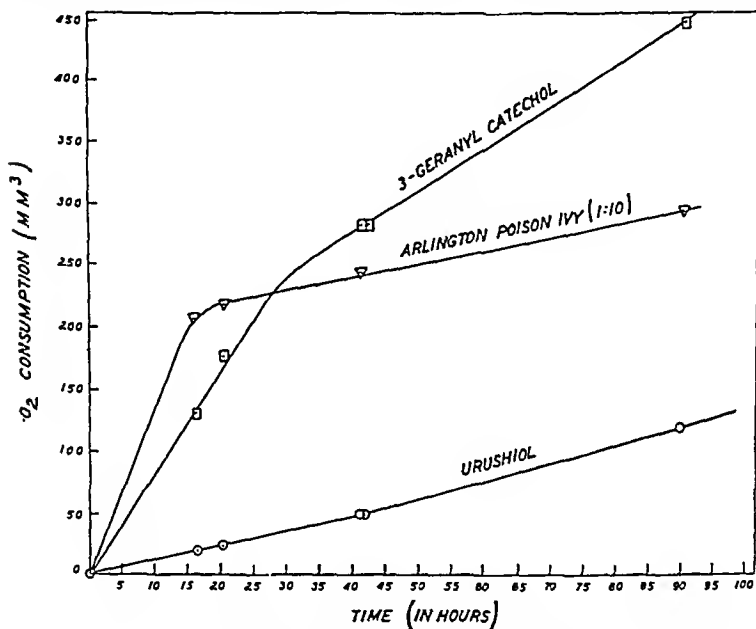


FIG. 4. AUTOXIDATION OF TOXICANTS

The control flask contained 3.3 ml. buffer, the experimental flask contained 3.3 ml. buffer plus one of the following: 0.02 ml. 3-geranyl catechol; 1.0 ml. 10% Arlington poison ivy (evaporated to dryness); 0.02 ml. urushiol.

to poison ivy and related toxicants. In view of the fact that there is a tremendous variation in the toxicity of dermatitis-producing preparations, as well as a very great difference in sensitivity of individuals to these materials, it was necessary to consider these factors in selecting the dosage to be employed in the patch test. If too little material was applied, no results were obtained, because no dermatitis appeared in either area. If too much material was used, no difference was observed, because a very severe dermatitis, which could not be differentiated, was produced in both areas. If amounts of toxicant just above the threshold of sensitivity were employed, clear-out positive results were usually obtained. The control area usually showed a much greater irritation; in many experiments no trace of symptoms appeared in the experimental area.

The technic employed in these studies was a modified patch test involving the use of a glass ring 1.75 cm. in diameter and 0.5 cm. high. The ring was held firmly against the skin and an aliquot (usually 0.1 ml.) of the ether extract placed in the ring. The solvent was evaporated with a current of air and the ring removed. The toxicant was left on the skin for 24 hours or until dermatitis was apparent, at which time the residual toxicant was removed with soap solution or organic solvent. Control and experiment areas were always treated identically. The severity of the dermatitis which appeared in both areas was

TABLE 1

Skin patch tests on humans comparing the dermatitis-producing properties of untreated and tyrosinase-treated toxicants

SUBSTRATE	POSITIVE REACTIONS	NEGATIVE REACTIONS	SUB-THRESHOLD REACTIONS	TOTAL EXPTS
Unprocessed poison ivy (Lederle)	1	0	7	8
Processed poison ivy (19.1%) (Lederle)	5	1	0	6
Poison ivy (2%) (Squibb)	0	0	2	2
Poison ivy (10%) (Arlington)	1	0	0	1
Poison ivy (0.1%) (Arlington)	0	0	1	1
Poison oak (5%) (Pitman-Moore)	0	0	5	5
Poison ivy oleoresin (10%) (Sharp & Dohne)	4	0	0	4
Poison ivy oleoresin (10%) (Mulford)*	4	0	2	6
Poison ivy oleoresin (10%) (Wyeth)	1	0	0	1
Kiurushi	1	0	0	1
Solvent Extracted Cashew Nut Shell Liquid (Irrington)	1	0	0	1
Cardanol (redistilled)	0	1	0	1
Urushiol*	6	1	0	7
3-geranyl catechol	3	0	0	3
Anacardol	2	1	0	3
Anacardic acid	2	0	0	2
Tetrahydro-anacardol (10%)	0	0	2	2
Tetrahydro-anacardic acid (10%)	0	0	1	1

* This includes the cup and gauze patch test experiments in which tyrosinase and toxicants were applied simultaneously to the skin.

compared for the next few days with reference to erythema, edema, spreading, pruritis, vesicle formation and, in severe cases, loss of epidermis and scarification.

Human experiments. The forearm or leg was used for the application of test material in 55 experiments, all but one of which were performed on males. The purpose of these skin tests was to determine the activity of tyrosinase on the dermatitis-producing properties of each of the available toxic preparations, rather than to accumulate many results with a single toxicant. The diagnosis of the comparative dermatitis of the control and experimental areas was always made by at least two (frequently several) observers. In several experiments colored photographs were taken of both areas. The human experiments are summarized in table 1. For positive results the control area is more severe

than the experimental while for negative results the control area is no worse than the experimental. Sub-threshold results mean that dermatitis was absent from both areas because concentrations of toxicants used were so weak that no dermatitis was produced. It is apparent from the table that in many experiments with a variety of different kinds of toxicant preparations the oxidation of the toxicant by tyrosinase has very considerably reduced the dermatitis-producing properties of the irritant preparation. Although the tyrosinase catalyzed the oxidation of tetrahydro-anacardol and tetrahydro-anacardic acid, like their non-hydrogenated homologues, the two hydrogenated compounds proved to be non-irritating either before or after treatment with tyrosinase. These results on the decrease in toxicity after hydrogenation are consistent with the work of Toyama (7), Landsteiner and Jacobs (21) and Keil and co-workers (6). As would be expected, most of the other preparations listed in table 1, which in sub-threshold concentrations produced no dermatitis, gave positive results with greater amounts of toxicant. Similarly, many of those preparations yielding negative results, because dermatitis was very severe in both areas, produced positive results when smaller amounts just above threshold were applied to the skin.

Since in all previous experiments the poison ivy or related toxicant has been inactivated by tyrosinase *in vitro* and then tested on the skin, the question arises as to whether or not the active principle can be inactivated on the human skin by this enzyme. Two sets of experiments were performed using on the experimental area 0.2 ml. Mulford 10% poison ivy oleoresin solution, 0.4 ml. tyrosinase and 0.4 ml. buffer. The control was the same except that inactivated (boiled) enzyme was used. In a second pair of experiments the experimental area was covered with 0.05 ml. urushiol, 0.4 ml. enzyme and 0.1 ml. buffer, while the control area was the same except that buffer replaced the enzyme. The solutions were either contained within glass cups or absorbed on gauze and held in close contact with the skin for not more than four hours with water-proof tape. The areas were thoroughly washed free of all reagents at the end of this time and then observed for dermatitis. In all four experiments the dermatitis of the control area was much the more severe of the two, indicating that poison ivy toxicants, while in contact with the skin, can be partially inactivated by tyrosinase. In one experiment 0.1 ml. Mulford 10% poison ivy oleoresin was left in contact with two skin areas for 4 hours and in 4 experiments for 12 hours and then washed off. A gauze patch containing 0.3 ml. tyrosinase plus 0.5 ml. buffer was then placed on the experimental area, while the control was covered with a patch containing 0.8 ml. buffer. The dermatitis which developed in the control area was more severe in these studies (except for one 12-hour experiment), indicating that tyrosinase is effective in decreasing the dermatitis even when the toxicant has been on the skin for 4-12 hours and after all the superficial toxicant has been washed from the surface of the epidermis.

Guinea pigs. Poison ivy dermatitis is similar but not identical in guinea pigs and man (21). Since guinea pigs are much less susceptible, they must first be plucked and sensitized before using. This technique, with minor variations, has

been utilized by all workers for poison ivy experiments (21-31). The procedure followed in this study was an adaptation of the methods used by these investigators. The flank was utilized as the area for the two patches, and 24 or more hours previous to the time of sensitization or administration of the experimental and control fluids the guinea pig was plucked by hand and the few remaining hairs removed by shaving. After the disappearance of all irritation from the epilation process, 0.1 ml. of 10% poison ivy oleoresin solution (in acetone) was applied to one flank in the sensitization process, while 0.1 ml. respectively of the control and experimental ether extracts were administered to the other flank in the actual investigation. The patch test technic was the same as that applied to humans. Thirty-one experiments with guinea pigs are summarized in table 2. Although guinea pigs are less sensitive than humans, it is apparent from the table that results are very similar, indicating an oxidative inactivation of poison ivy toxicants by tyrosinase.

TABLE 2

Skin patch tests on guinea pigs comparing the dermatitis-producing properties of untreated and tyrosinase-treated toxicants

SUBSTRATE	POSITIVE REACTIONS	NEGATIVE REACTIONS	SUB-THRESHOLD REACTIONS	TOTAL EXPTS
Processed poison ivy (19.1%) (Lederle)	0	2	0	2
Poison ivy (10%) (Arlington)	1	1	1	3
Poison ivy oleoresin (10%) (Sharp & Dohme)	8	1	0	9
Urushiol	5	1	0	6
3-geranyl catechol	1	1	2	4
Anacardol	2	1	0	3
Anacardic acid	2	1	1	4

(4) *Colorimetric studies on the oxidation of poison ivy toxicants by tyrosinase.* Since the oxidation of most phenolic substrates to quinones by tyrosinase is accomplished by the production of pigmented compounds, it seemed possible that a similar reaction might occur with poison ivy toxicants. Qualitative data were obtained in each experiment which showed that during the progressive action of tyrosinase on the various toxicants there was a gradual darkening of the solution. The control also showed a slight darkening after many hours, indicating that some autoxidation had occurred. In view of the fact that all the studies on the action of tyrosinase upon the toxicants suggest that phenolic hydroxyl groups are converted to quinones, it should be possible to demonstrate this by a chemical test for phenols. The 2,6 dibromquinonechlorimide test (22) for phenols was found unsatisfactory, although it did give some evidence of a decrease in concentration of phenolic groups of the toxicants produced by tyrosinase. The ferric chloride test, yielding a blue color for phenolic groups (33), proved reliable and when used, clearly demonstrated a distinctly greater amount of phenolic groups in the untreated solution of toxicant than in the solution of toxicant treated with tyrosinase. This was typical of all toxicants

tested except anacardic acid and its derivatives, which revealed no such difference. In qualitative experiments the ferric chloride test indicated a progressive decrease in amount of phenolic substance in the toxicant solution due to the action of tyrosinase. At the present time it would appear that the ferric chloride test for the action of tyrosinase is neither as sensitive nor as quantitative as the manometric method.

DISCUSSION. Since poison ivy toxicants and related materials have been shown to be phenolic compounds (1-7), it is not surprising that the toxicants resemble many other phenolic derivatives in undergoing at 37°C. a slow autooxidation to quinones with an accompanying darkening of color. Similarly, like many other phenolic compounds the oxidation of these irritants, as measured manometrically and colorimetrically, is markedly accelerated by the addition of mushroom tyrosinase (13). The rate of enzymatic oxidation, however, is determined by the specific molecular configuration of the toxicant undergoing oxidation (see figs. 1-2). Accompanying the enzymatic oxidation of these materials there is a very appreciable loss in their dermatitis-producing properties. This is comparable to the loss of activity resulting from the oxidation of certain hormones such as adrenalin and estrogens by tyrosinase (10).

Experiments on the use of tyrosinase (or other phenolases such as laccase (11, 12) in the treatment of poison ivy dermatitis are indicated in view of the fact that the partial inactivation of poison ivy toxicants on the human skin by tyrosinase has been successful.⁴ Even when the poison ivy extract was applied to the skin four and twelve hours before the enzyme, favorable results were obtained. These experiments suggest the possible use of tyrosinase as a prophylactic measure before or after contact with the toxicant, but before symptoms of dermatitis have appeared. If this phenolase is effective even after erythema has first appeared, then the use of tyrosinase in the treatment of poison ivy dermatitis is also suggested in view of the innocuous nature of the enzyme. While tyrosinase may be useful in treating dermatitis in its early stages, it seems doubtful whether it will be very effective in treating the advanced stages after the toxicants have passed through the epidermis and dermis and have reached the lymphatics (29, 34). It may be possible, however, to facilitate penetration of the enzyme into the skin by increasing its permeability with "spreading factor" (hyaluronidase) (35, 36), by the utilization of electrophoretic methods (37, 38) or by other means such as intradermal injection of sterile tyrosinase in the area showing dermatitis.

SUMMARY

Purified mushroom tyrosinase catalyzes the oxidation of the toxic principles of a large number of different commercial poison ivy concentrates, as well as extracts of related materials from poison oak, Japanese lac and cashew nut shell liquid. It has a similar action upon the purified toxicants: urushiol, 3-geranyl catechol, anacardol, tetrahydro-anacardol, anacardic acid, tetrahydro-anacardic

⁴ Any possible effect of the many plant juices recommended for the treatment of poison ivy (see McNair, 2) might be explicable in terms of the tyrosinase present

acid and cardanol. These irritant materials slowly autoxidize, even in the absence of tyrosinase. The action of tyrosinase in the oxidation of the phenolic groups of these toxicants has been studied by oxygen consumption measured manometrically, by a darkening in color during oxidation and by a disappearance of phenolic groups reactive with ferric chloride.

Accompanying the oxidation of these toxic materials by tyrosinase there is an appreciable decrease in the dermatitis-producing properties as measured by using both human and guinea pig skin patch tests. Tyrosinase is effective in the inactivation of the toxicants not only *in vitro* but also when applied to the human skin simultaneously with the toxicants or a few hours after they have penetrated into the skin. The use of tyrosinase in the treatment of poison ivy dermatitis in its early stages is suggested, although no information is as yet available concerning the utilization of this enzyme for the inactivation of the toxicants in the more advanced stages of the dermatitis.

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THE ISOLATION OF DI(p-CHLOROPHENYL) ACETIC ACID (DDA)
FROM THE URINE OF RABBITS POISONED WITH 2,2 BIS
(p-CHLOROPHENYL) 1,1,1 TRICHLORETHANE (DDT)

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In a previous communication (1), the isolation and identification of 2,2 bis (p-chlorophenyl) 1,1,1 trichlorethane from both the urine and feces of rabbits having received this substance intragastrically was reported.

In that report it was pointed out that the urine of DDT poisoned rabbits after acidification and extraction with ether yielded two fractions each containing organically bound chlorine. One of these was ether soluble and water insoluble. The other was ether soluble when extracted from an acid medium, but became water soluble when made alkaline. It is the latter fraction that forms the basis of this report.

EXPERIMENTAL. In general the experimental conditions outlined in the previous report (1) were also maintained in this one. A mixed group of both male and female rabbits, weighing from 1.9 to 2.5 kgm. were given repeatedly over a period of 33 days, 240 mgm. per kgm. DDT as an 8% solution in olive oil by stomach tube at one to three day intervals, depending upon the severity of the symptoms. While only one animal survived two such doses, two survived a maximum of nine doses and the remaining nine a varying number between these two extremes. Some replacements were made in those instances in which animals died following a relatively small number of administrations. Three animals were still receiving DDT at the time the experiment was terminated.

The urines were collected daily, pooled, acidified, and extracted with ether, as described previously (2). The semi-viscous residues of the evaporated ether extracts were stored in a desiccator until after all of the DDT had been given. Any feces contaminated specimens were discarded.

The total urine extract residues obtained during the last 27 days of DDT administration, representing 8,693 cc. urine, were pooled. These were taken up in absolute alcohol and made up to a total volume of 250 cc. Analysis of an aliquot by reduction with sodium in absolute alcohol as outlined in a previous publication (2) showed there was a total of approximately 800 mgm. of organic chlorine in this alcoholic solution. The balance of the alcoholic extract containing 796 mgm. chlorine was evaporated on a steam bath under a current of air to a dark almost black viscous residue of about 12 cc. This residue was then taken up in 350 cc. alcohol-free ether. Considerable ether insoluble material, which also was water insoluble under these conditions was discarded. Analysis of this fraction yielded only 7.5 mgm. chlorine.

The 350 cc. ether solution was next washed 5 times with distilled water, using 20 cc. in each of the first 3 washings, and 10 cc. in each of the last two. Analy-

sis of the evaporated residue showed the absence of all but mere traces of chlorine.

The washed ether solution was evaporated to a solid residue with approximately 4 cc. of an oily supernatant layer. To this was added 1 gm. of Na_2CO_3 in 10 cc. distilled water. Immediate reaction followed with some evolution of gas, and the formation of a thick viscous residue. This residue was next transferred to a separatory funnel with the aid of distilled water and alcohol-free ether. Sufficient distilled water was added to make total a volume of 100 cc. More ether was added to a volume of 100 cc. More Na_2CO_3 was added until a pH of 8.4 was reached. After 4 extractions of the aqueous solution with ether the combined ethers were washed 4 times, 5 cc. of distilled water being used in each washing. On analysis these pooled washings contained 0.6 mgm. chlorine. The combined ethers (Fraction I) were evaporated to a moderate amount of viscous reddish brown residue. The residue was dissolved in absolute alcohol and an aliquot analyzed. This fraction had a total of 199 mgm. organic chlorine.

The heavily pigmented alkaline aqueous solution was acidified with glacial acetic acid to pH 4.8. This was extracted 4 times with 75 cc. alcohol-free ether each. The pooled ethers were then washed five times with 10 cc. of distilled water for each washing. Analysis of the combined washings and the extracted aqueous solution, after evaporation yielded 4.2 mgm. chlorine.

The ether extracts were pooled, evaporated, and the residue taken up in 100 cc. absolute alcohol. Analysis of an aliquot showed this (Fraction II) contained approximately 583 mgm. chlorine. Approximately one third of this was subjected to 27 recrystallizations from hot 80% alcohol. A crystalline powder weighing 69 mgm. was finally obtained. These crystals had a melting point of 165-166°C. After drying to constant weight a micro combustion analysis by Dr. A. T. Ness of the chemistry laboratory of this Institute, gave C 59.94; H 3.69; Cl 25.09; Theoretical C 59.81; H 3.59; Cl 25.23. The analytical data agree well with those given by Grummit and associates for di(p-chlorophenyl)-acetic acid which they synthesized (3).

Another aliquot of the alcoholic solution containing some 325 mgm. organic chlorine was evaporated to dryness. The residue was dissolved in about 40 cc. boiling 36% acetic acid, boiled for a few minutes with 1 gram norite to decolorize, filtered and washed with a little boiling 36% acetic acid. On cooling a crop of colorless crystals deposited (fig. 1). These were filtered, washed with water and dried in vacuo over a saturated solution of NaOH. The dried crystals weighed 315 mgm. They gave a melting point of 165-166°C., and a chlorine determination showed 24.94%. The crystallization from acetic acid was adapted from a procedure used by Dr. H. Bauer in this laboratory in the preparation of DDA from DDT. Spectrophotometric analysis of this material after nitration (4) showed a maximum absorption at 540 μ , and gave an optical density curve between the wave lengths of 460 and 620 millimicrons identical, within experimental error, with that of the pure compound made from DDT (fig. 2).

DISCUSSION. Summarizing the more essential data the following should

benoted: 1. Of a total of 796 mgm. of organically bound chlorine extracted from the urine of DDT poisoned rabbits, 199 mgm. or about 25% were recovered as an ether soluble—water insoluble fraction which according to the analytical data in a previous paper (1) should be regarded as unchanged DDT. 2. The



FIG 1 PHOTOMICROGRAPH OF DDA CRYSTALS ISOLATED FROM FRACTION 2 BY CRYSTALLIZATION FROM 36% ACETIC ACID

remaining 75% of the organically bound chlorine, 583 mgm. in all, was characterized by its solubility in ether as an acid, and solubility in water as a sodium salt. From one-third of this fraction 69 mgm. of di(*p*-chlorophenyl) acetic acid were isolated by repeated recrystallizations from 80% alcohol, yield of a little over 8%. 3. From the remainder 315 mgm. of the same crystalline material were obtained by one crystallization from 36% acetic acid with a yield of

about 24%. All this is consistent with the recently reported isolation of this substance from the urine of DDT poisoned rabbits by White and Sweeney (5).

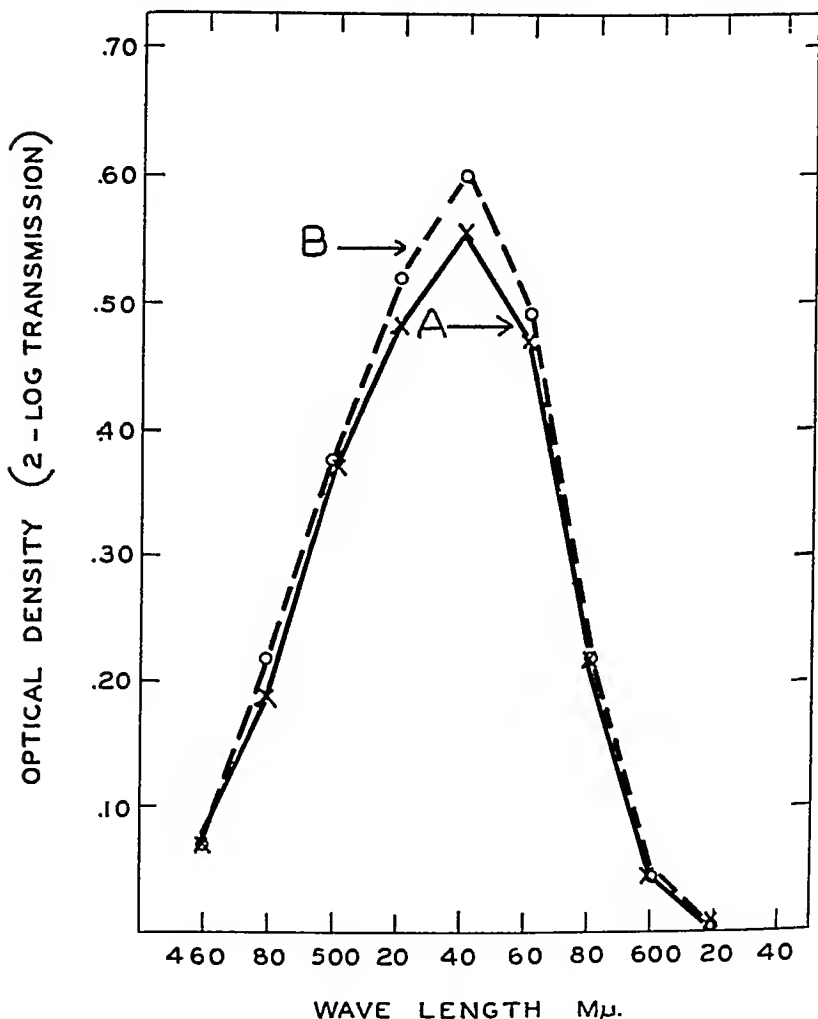


FIG. 2. ABSORPTION CURVE OF NITRATED DDA

Optical density plotted against wave length in millimicrons. All transmission measurements made at a depth of 10 cm. with 0.1 mg. DDA equivalent in 5 cc. benzol and 10 cc. sodium methylate solution. Curve A pure DDA made from DDT, curve B DDA isolated from fraction 2.

It is of course probable that by improving the technique of isolation a better yield of the acetic acid compound may be obtained, but it is also possible that other degradation products, partially or even completely dechlorinated, may

yet be present in addition to the two already isolated and identified. In the identification of partially dechlorinated degradation products the method of organic chlorine determination previously described (2, 6) will continue to be a useful guide; the identification of completely dechlorinated degradation products of DDT, if such there are, will be a more difficult task.

SUMMARY

Di(p-chlorophenyl) acetic acid has been isolated from the urine of rabbits given DDT orally. The physiological significance of this degradation product in the detoxification of DDT is under investigation.

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A COMPARATIVE STUDY OF THE EFFECTIVENESS OF 1,3,7-TRIMETHYLYXANTHINE AND CERTAIN DIMETHYLYXANTHINES (1,3-DIMETHYLYXANTHINE AND 3,7-DIMETHYLYXANTHINE) AGAINST FATIGUE

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In a previous publication from this department (Huidobro and Amenabar) there were reported the findings of a study of the effectiveness of caffeine (1,3,7-trimethylxanthine) against fatigue. The conclusion was reached that the drug lowers the excitatory threshold of acetylcholine in the action of this substance on the neuromuscular junction. It was thought worthwhile to study the effectiveness of certain dimethylxanthines against fatigue in comparison with this property of caffeine, particularly in view of the fact that it is stated in the literature that dimethylxanthines have a stimulating effect on skeletal muscle, although their mode of action is not clarified (Goodman and Gilman, 1941).

METHODS. The animals used were cats anesthetized with sodium pentobarbital (Nembutal Abbott 0.033 gm dissolved in 1 cc of 25% urethane per kilo body weight, intraperitoneally). The trachea was cannulated so that artificial respiration might be administered if necessary.

The investigations were made on the quadriceps femoris muscle. The two ends of the femur were fixed with clamps and the tendon of the quadriceps was attached to the short arm of a lever which worked against rubber bands. The muscle was stimulated indirectly through its nerve, which had been previously isolated and sectioned. The stimuli consisted of discharges from condensers controlled by vacuum tubes. Only maximum stimuli were used. The electrodes were made of silver insulated with rubber.

Drugs used in the experiments were Prostigmine (Roche), certain salts of 1,3-dimethylxanthine (theophyllin), theophyllin ethylenediamine (Aminophyllin, Collier¹) in 3% aqueous solution and theophyllin sodium acetate (Bayer) in 4% aqueous solution, a salt of theobromine (3,7-dimethylxanthine), namely theobromine sodium salicylate (Diuretin) in 5% aqueous solution, and caffeine (1,3,7-trimethylxanthine) 2.80 gm dissolved in 100 cc of 4% sodium benzoate. (For reasons of convenience in this presentation, these substances, in the described concentrations, will be referred to as aminophyllin, theophyllin, diuretin, and caffeine.) In this concentration the solution of trimethylxanthine is equivalent to the preparations of dimethylxanthines employed. These concentrations of the dimethylxanthines were used because of the percentages of theophyllin and theobromine which had been studied by other investigators (Goodman and Gilman, 1941). The drugs were injected into the abdominal aorta after a preliminary ligation of the contralateral common iliac artery and of the median sacral artery. Brazilian curare was also used, it was injected into the external jugular vein.

RESULTS I.—Action of diuretin, theophyllin, aminophyllin, and caffeine on the contraction of directly stimulated muscle. All of these drugs increase the

¹ Appreciation is expressed to Roche and Collier for the gift of aminophyllin.

tension developed by the muscle when they are injected during muscular stimulation. Their effectiveness, however, varies quantitatively as the frequency of stimulation is varied.

a) Low frequencies, up to 150 per minute. Diuretin is the one which has the weakest action. When 0.50 to 0.60 cc. of a 5% solution of this drug was injected into the abdominal aorta it failed in some animals to alter the muscular tension at all (fig. 1). In others it produced a slight increase of the amplitude of contraction.

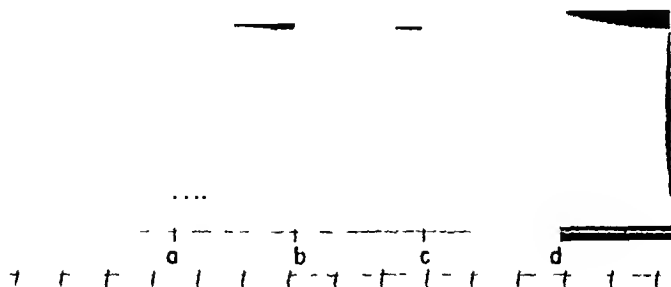


FIG. 1. EFFECT OF DIURETIN, AMINOPHYLLIN, THEOPHYLLIN, AND CAFFEINE UPON MUSCULAR CONTRACTION UNDER INDIRECT STIMULATION OF THE NERVE AT LOW FREQUENCY (155 PER MINUTE)

Upper tracing: quadriceps muscle. Upper line: a) intra-aortic injection of 0.5 cc. of diuretin; b, intra-aortic injection of 0.5 cc. of aminophyllin; c, intra-aortic injection of 0.5 cc. of theophyllin, d, intra-aortic injection of 0.5 cc. of caffeine. Lower line: time in minutes.

Theophyllin, as well as aminophyllin and caffeine, when injected into the abdominal aorta in dosages of 0.50 to 0.60 cc. produced an increase of the muscular tension in all the animals studied (fig. 1). In some cases the increase was slight. When these drugs are listed in the order of their strengthening action on the development of tension in indirectly stimulated muscle, from the least to the most effective, the order is as follows: diuretin, theophyllin, aminophyllin, and caffeine (fig. 1).

b) Higher frequencies, up to 20 per second. All of the substances studied, when injected into the abdominal aorta in dosages of 0.50 to 0.60 cc., produced a definite increase in the amplitude of the tension developed by the muscle, as can be seen in figure 2. When their effectiveness is again compared, the order which we had established for the low frequencies of stimulation is changed, as theophyllin is more effective in this range than aminophyllin. The difference between them, however, is slight (fig. 2). In all the cases studied, the strength-

ening effect was of some importance and by no means slight, as it had been in some of the cases of low frequency stimulation.

II.—*Effect of diuretin, theophyllin, aminophyllin, and caffeine on the action of prostigmine.* The amount of prostigmine injected into the abdominal aorta fluctuated in the various animals studied from 85 to 400 mgm. The effects which the drugs under investigation had upon the action of prostigmine was more dependent upon the frequency of stimulation than upon the dose of prostigmine used.

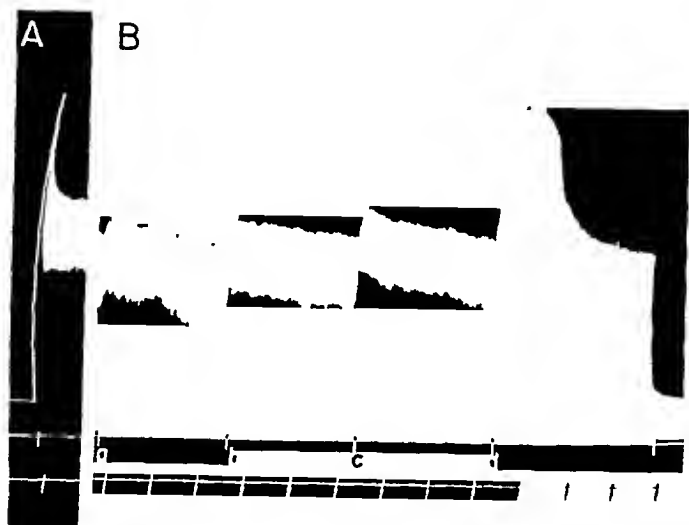


FIG. 2. EFFECT OF DIURETIN, AMINOPHYLLIN, THEOPHYLLIN, AND CAFFEINE ON MUSCULAR CONTRACTION UNDER INDIRECT STIMULATION OF THE MUSCLE WITH A HIGH FREQUENCY (20 PER SECOND)

Upper tracing quadriceps muscle

Lower line, time in minutes

A.—Upper line signal, onset of stimulation. Lower line, time in minutes
 B—Four minutes after A. Upper line a, intra-aortic injection of 0.5 cc. of diuretin;
 b, intra-aortic injection of 0.5 cc. of aminophyllin, c, intra-aortic injection of 0.5 cc. of
 theophyllin, d, intra-aortic injection of 0.5 cc. of caffeine, signal, end of stimulation.
 Lower line time in minutes

a) When the muscle was stimulated with frequencies lower than 100 per minute (very low frequencies were not studied), the effect of the prostigmine, whatever its dosage, was altered differently according to the substance used. Under these conditions diuretin consistently produced a pronounced increase in the muscular tension accompanied as well by an elevation of the base line of the muscular contractions (fig. 3). Theophyllin had the same effect as diuretin though not as marked (fig. 3). The other drugs, aminophyllin and caffeine, increased the depressant action of the prostigmine, although the effect of the aminophyllin was at times only scarce.

b) When higher frequencies were used, up to 300 per minute, the effect was different. All four drugs exaggerated the depressant action of prostigmine,

but diuretin and aminophyllin did not produce a development of tension. A typical experiment is presented in figure 4. In this figure it can be observed that caffeine has an effect far superior to that of the dimethylxanthines, in so far as intensity of action is concerned. In this type of experiment it is not possible to ascertain which of the dimethylxanthines is the most active.

When small doses of prostigmine were used and the drug did not markedly decrease muscular contraction, diuretin, and more rarely, theophyllin, failed in some animals to augment the depressant effect of prostigmine at all.

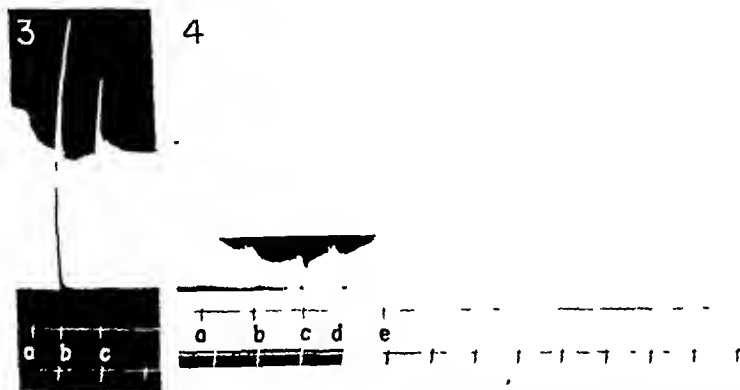


FIG. 3 EFFECT OF DIURETIN AND THEOPHYLLIN ON THE ACTION OF PROSTIGMINE UPON THE QUADRICEPS MUSCLE STIMULATED INDIRECTLY AT A FREQUENCY OF 100 PER MINUTE

Upper tracing: muscle. Upper line: a., intra-aortic injection of 85 micrograms of prostigmine, b, intra-aortic injection of 0.50 cc. of diuretin; c, intra-aortic injection of 0.50 cc. of theophyllin. Lower line, time in minutes

FIG. 4 EFFECT OF THEOPHYLLIN, AMINOPHYLLIN, DIURETIN, AND CAFFEINE ON THE ACTION OF PROSTIGMINE UPON THE QUADRICEPS MUSCLE STIMULATED INDIRECTLY AT A FREQUENCY OF 200 PER MINUTE

Upper tracing: muscle. Upper line: a, intra-aortic injection of 25 micrograms of prostigmine (The animal had received previously a 250 microgram injection of prostigmine intra-aortically), b, intra-aortic injection of 0.50 cc. of aminophyllin, c, intra-aortic injection of 0.50 cc. of theophyllin; d, intra-aortic injection of 0.50 cc. of diuretin, e, intra-aortic injection of 0.50 cc. of caffeine. Lower line: time in minutes

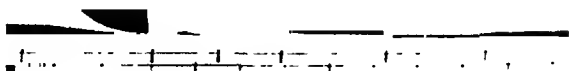
III.—Effect of diuretin, theophyllin, aminophyllin, and caffeine on the action of curare. The dimethylxanthines, like the trimethylxanthines, have a distinct decurarizing effect, although the intensity of action is not equal for all of them. When the animal had received a strong dose of curare as in the experiment represented in figure 5 we could observe clearly the comparative action of the drugs. Under these circumstances aminophyllin is inactive, theophyllin only slightly so, diuretin visibly active and caffeine very active. This does not mean that aminophyllin is entirely inactive against curare, for, if the curarizing dose itself is appropriately small, as shown in figure 6, this drug does have a distinct decurarizing effect.

DISCUSSION. When one compares the effectiveness of the dimethylxanthines against fatigue with the effectiveness of the trimethylxanthines, one is

led to believe that the differences are quantitative rather than qualitative, although the action of the latter is far superior to that of the former.

In a former study carried out in this department concerning the action of 1,3,7,trimethylxanthine, it was concluded that caffeine has the property of lowering the excitatory threshold of acetylcholine (Huidobro and Amenabar). It is supposed that for the same reasons applied in the former study, the case

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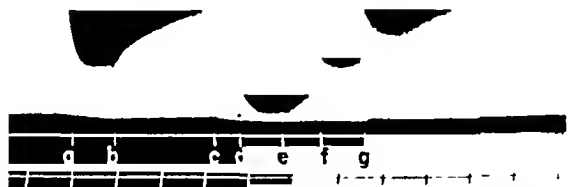


FIG. 5. EFFECT OF DIURETIN, AMINOPHYLLIN, THEOPHYLLIN, AND CAFFEINE ON THE ACTION OF CURARE. UPPER TRACING, QUADRICEPS MUSCLE STIMULATED INDIRECTLY AT A FREQUENCY OF 100 PER MINUTE

Upper line: first signal (from left to right), intravenous injection of curare; second signal, intra-aortic injection of 0.50 cc. of diuretin; third signal, intra-aortic injection of aminophyllin; fourth signal, intra-aortic injection of 0.50 cc. of theophyllin; fifth signal, intra-aortic injection of 0.50 cc. of caffeine; sixth signal, intra-aortic injection of aminophyllin. Lower line: time in minutes.

FIG. 6. EFFECT OF AMINOPHYLLIN, DIURETIN, THEOPHYLLIN, AND CAFFEINE ON THE ACTION OF CURARE. UPPER TRACING, QUADRICEPS MUSCLE STIMULATED INDIRECTLY AT A FREQUENCY OF 120 PER MINUTE

Upper line: a., curare intravenously; b., 0.50 cc. of aminophyllin intra-aortically; c., curare intravenously; d., 0.50 cc. of diuretin intra-aortically; e. 0.50 cc. of aminophyllin intra-aortically; f., 0.50 cc. of theophyllin intra-aortically; g., 0.50 cc. of caffeine intra-aortically. Lower line: time in minutes.

of the dimethylxanthines is similar, and that they have a quantitatively smaller but identical action on the excitatory threshold of acetylcholine.

Huidobro and Amenabar (1944) showed that the intra-arterial injection of caffeine, in doses of 0.007 to 0.035 gm. produces a development of tension in resting muscle in some experiments. During the present investigation we noted the same response in some instances when a dose of 0.014 gm. was injected. When similar doses of the dimethylxanthines were used we did not at any time

observe a development of tension in unstimulated muscle. This does not mean necessarily that in larger doses the dimethylxanthines would not produce such a muscular tension; in fact there are reasons for supposing that they would, as indicated below. In the publication referred to above it was noted that the development of tension in indirectly stimulated muscle was more manifest and more frequently seen when the injection of caffeine was preceded by a dose of prostigmine. We have again observed this fact, as shown in figure 4. When this definite action of caffeine is compared with the effect of the dimethylxanthines it is evident that some of these drugs behave differently; i.e., diuretin and theophyllin have an action quite different from that of aminophyllin (see Section II, Results). When muscle is being stimulated indirectly at low frequencies, diuretin and theophyllin, injected after prostigmine, consistently produce an intense and brief development of tension (fig. 3). This does not occur on the injection of aminophyllin nor does it occur in the case of any of these drugs when the muscle is being stimulated at high frequencies (fig. 4). By considering figure 3 one might be led to think that diuretin and theophyllin have a brief, powerful anti-prostigmine effect. In reality this is not the case. The exaggerated increase of tension produced by these drugs actually masks an important factor; namely, the fact that the amplitude of the muscular contractions is decreased. These two drugs, then, do not differ from the others in the matter of augmenting the depressant action of prostigmine, but rather, they differ in that these two, after a prostigmine injection, when muscle is being stimulated at low frequencies, are able to produce a strong development of tension which aminophyllin does not produce and which none of these substances are able to produce when the frequency of stimulation is higher. We believe that this development of tension resultant from diuretin and theophyllin is due to a direct action on the muscle, and that it could be observed in the unstimulated muscle if the quantity of the drugs used were much greater.

Goodman and Gilman (1941) affirm that the xanthines have a stimulating action on skeletal muscle, the strength of their action being, from greatest to least: theobromine, caffeine, and theophyllin. Our results do not concur with those indicated by Goodman and Gilman. We have found that among the drugs which were studied the ones which lower the excitatory threshold of acetylcholine are, in the order of the strength of their action: caffeine, theophyllin, and theobromine. We cannot explain this discrepancy, as the authors make their statement without further elaboration.

Like caffeine, theobromine and theophyllin are insoluble substances. In this study caffeine was dissolved in a 4% solution of sodium benzoate. In the cases of the other two drugs, certain soluble salts were used: theobromine sodium salicylate, theophyllin sodium acetate, and theophyllin ethylenediamine. In view of this fact the suspicion might exist that the effects of caffeine, theobromine, and theophyllin were not produced by them but by the sodium benzoate, salicylate and acetate and by ethylenediamine. In another study it was shown that sodium benzoate is inert (Huidobro and Amenabar). We believe that the similarity of action of these drugs viewed in the light of their chemical

similarity is evidence enough to attribute the experimental results to them rather than to the sodium salicylate, sodium acetate and ethylenediamine, substances of markedly differing composition.

SUMMARY

In cats anesthetized with nembutal a comparative study was made of the actions of 1,3,7 trimethylxanthine (caffeine) and of certain salts of 1,3 dimethylxanthine (theophylline) and 1,7 dimethylxanthine (theobromine), namely theophyllin sodium acetate, theophyllin ethylenediamine, and theobromine sodium salicylate. The study was made of their effects on the quadriceps femoris muscle stimulated indirectly at various frequencies (Section I), and of their effects on the action of prostigmine (Section II) and of curare (Section III).

It was found that all of these drugs have some effectiveness against fatigue and the differences between them are only quantitative. When listed according to the intensity of their action, from least to greatest, they appear thus: at low frequencies: diuretin (theobromine sodium salicylate), theophyllin (theophyllin sodium acetate), aminophyllin (theophyllin ethylenediamine), and caffeine (fig. 1); at higher frequencies: diuretin, aminophyllin, theophyllin, and caffeine (fig. 2).

The effect of these drugs is summated with that of prostigmine (see fig. 4), but diuretin and theophyllin at low frequencies of stimulation produce an intense development of muscular tension (see fig. 3 and Discussion).

All the drugs investigated have a decurarizing action and their effectiveness is in ascending order: aminophyllin, theophyllin, diuretin, and caffeine (see figs. 5 and 6).

The mechanism of their action against fatigue is discussed and it is believed that they have the property of lowering the excitatory threshold of acetylcholine at the level of the neuromuscular junction.

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PHARMACOLOGICAL STUDIES OF SOME BENZOFURANONE DERIVATIVES

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The search for an antispasmodic drug which would have both a marked musculotropic and neurotropic action but without the undesirable side effects of atropine has promoted the investigation of many diverse types of compounds. This publication deals with the pharmacology of some benzofuranone derivatives,¹ with particular reference to their antispasmodic effects.² Chemically, this series is a new type which has apparently not been investigated previously. Table 1 gives the formulae of a number of benzofuranones and includes some of the pharmacological properties of the series. Intraperitoneal toxicity was determined in mice. The antispasmodic action was studied on the isolated rabbit small intestine by the usual technique. Contraction of the muscle was produced by adding either 20 micrograms acetylcholine or 25 mgm. barium chloride to the 100 cc. of Ringer-Locke solution in the muscle bath. Atropine sulfate in amounts of 0.5 to 1 microgram usually brought about a moderate to complete relaxation of the spastic contraction induced by acetylcholine, while 1 to 2 mgm. papaverine HCl similarly relaxed the muscle spasm evoked by barium chloride. After the response of a particular muscle strip to acetylcholine or papaverine was determined, different amounts of the antispasmodic under investigation were added and the responses compared with those produced by atropine and papaverine. In the table the value of 1.0 was assigned to atropine sulfate as a relaxing agent against acetylcholine spasm, and, similarly, papaverine was considered to have the value 1.0 against bariumchloride spasm. The relative activities of the antispasmodics under investigation are expressed as fractions or multiples of the standard value. Usually the atropine-like effect against acetylcholine is considered a measurement of the parasympathotropic action, and the papaverine-like action is referred to as the musculotropic action of the antispasmodic.

A consideration of table 1 makes possible a comparison of various pharmacological properties of the benzofuranone derivatives with atropine, papaverine and Trasentin. It is seen that none of the antispasmodics listed are as effective against acetylcholine-induced spasm as atropine, the best of the benzofuranones being approximately $\frac{1}{10}$ as active. However, against barium chloride spasm (musculotropic action) a number of these compounds were one and one-half to two times more effective than papaverine. In contrast, atropine has only one-half the activity of papaverine against the barium chloride spasm. Thus,

¹ These compounds were synthesized by Dr. A. W. Weston and Mr. W. B. Brownell of Abbott Laboratories, North Chicago, Illinois.

² A preliminary report appeared in the Federation Proceedings, 1945.

TABLE 1
Benzofuranone derivatives
(All compounds as hydrochlorides)

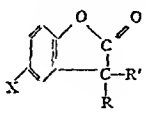
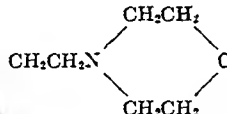
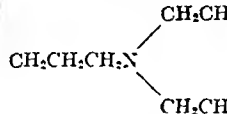
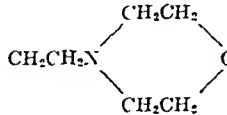
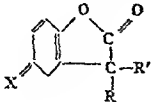
AP NO.				APT. LD ₅₀ (MICE I.P.)	ANTISPASMODIC ACTIVITY AGAINST		LOCAL ANESTHETIC EFFECT 1% SOL. ON RABBIT EYE	
	R	X	R'		Acetyl- choline	BaCl ₂	Dura- tion in minutes	Irrita- tion
43	C ₆ H ₅	H	CH ₂ CH ₂ N(C ₂ H ₅) ₂	mgm./ kgm. 205	1/10- 1/15	1.5- 2.0	30-80	+
45	C ₆ H ₅	H	CH ₂ CH ₂ N(CH ₃) ₂	125	1/15	1.5- 2.0	15	+++
52	C ₃ H ₇	CH ₃	CH ₂ CH ₂ N(C ₂ H ₅) ₂	230	1/25	1.0	20	+++
56	C ₆ H ₅	H		450	1/500	1/2	*	+++
58	C ₆ H ₅	H		200	1/25	1/2	50	+ to ++
59	C ₆ H ₅	H		175	1/10- 1/20	1.0		
60	C ₆ H ₅	CH ₃	CH ₂ CH ₂ N(C ₂ H ₅) ₂	150	1/30	1.5- 2.0	*	+++
66	C ₆ H ₅	H	CH-CH ₂ N(C ₂ H ₅) ₂ CH ₃	170	1/20	2.0	0	+
67	C ₆ H ₅	H	CH ₂ CH ₂ CH ₂ N(C ₂ H ₅) ₂	130	1/10	1.5	8	+++
72	C ₆ H ₅	H	CH ₂ CH-N(C ₂ H ₅) ₂ CH ₃	125	1/40	1-1.5	12	++

TABLE 1—Continued

AP NO				APP. LD ₅₀ (MICE I.P.)	ANTISPASMODIC ACTIVITY AGAINST		LOCAL ANESTHETIC EFFECT 1% SOL. ON RABBIT EYE	
	R	X	R'		Acetyl- choline	BaCl ₂	Duration in minutes	Irrita- tion
73	Cyclo— C ₆ H ₁₁	H	CH ₂ CH ₂ N(C ₂ H ₅) ₂	200	1/10– 1/15	1.5– 2.0	25	+++
81	C ₆ H ₅	H	CH ₂ CH ₂ CH ₂ N(C ₂ H ₅) ₂	250	1/30	1.0	45	+++
82	C ₆ H ₅	H	CH ₂ CH ₂ N(C ₄ H ₉) ₂	300	1/100	1.0	5	+++
132	C ₆ H ₅	H	CH ₂ (CH ₂) ₄ CH ₂ N(C ₂ H ₅) ₂	80	1/170	1/10	*	+++
	Trasentin			270	1/25	1.0	10	++
	Atropine sulfate			250	1.0	1/2		
	Papaverine HCl			225	1/1000	1.0		

* Too irritating to determine anesthesia.

some of the benzofuranones possess a remarkable musculotropic action as well as a substantial neurotropic effect.

A number of these compounds also show local anesthetic action, a property often associated with antispasmodic drugs (Crohn, et al. (1)). The duration of anesthesia induced by AP 43, AP 58, and AP 75 is comparable to that obtained with Butyn, but the latter is somewhat less irritating.

Among the more promising members of the group, 3(beta-diethylaminoethyl)-3-phenyl-2-benzofuranone HCl, in the following referred to by its code number AP 43, was studied most extensively. Reference is made also to AP 73, which contains the cyclohexyl ring instead of the 3-phenyl ring.

TOXICITY AND FATE OF AP 43. 1. Acute toxicity. Table 2 gives the data on AP 43 in mice obtained by various routes of administration, and also data on rabbits and dogs. In the latter species, the lethal dose was not determined, but the general effects of AP 43 by intravenous and oral route were observed. The general symptomatology of AP 43 in toxic doses is that of a convulsant. The animals become excited, tremble, jerk and finally tonic-clonic convulsions appear. Symptoms appeared immediately upon intravenous injection; after oral administration approximately 20–30 minutes elapsed before maximum symptoms became apparent. On fatal doses given intravenously, the animals die in a few minutes and with oral administration, within one or two hours. Delayed deaths occur rarely. The cause of acute death is re-

spiratory paralysis. Occasionally, the convulsive phase will be brief or even absent, and a marked depressant action becomes apparent. This was observed more frequently in dogs after oral administration than in any other species. As can also be seen from table 2, non-hypnotic doses of Nembutal permit dogs to tolerate otherwise convulsive doses of AP 43 without symptoms. In dogs vomiting occurred occasionally after the administration of large doses by mouth. The toxicity and symptomatology was essentially the same for AP 73 as for AP 43.

TABLE 2
Toxicity of AP 43

SPECIES	NO ANIMALS USED	MODE OF ADMINISTRATION	LD ₅₀ [*]	SYMPTOMS
			<i>mgm/kgm</i>	
Mice	167	i p	205	
	133	subcu	395	
	185	orally	660	
Rats	44	orally	600	
Rabbits	40	subcu	250	
	13	i v	23.2	
			Dosage	
Dogs	2	i v	5.0	No effect
	2	i v	10.0	1 slight convulsions
	4	i v	15.0-20.0	Convulsions, recovery
	3	i v	20 + 10-15 nembutal	No convulsions
	4	orally	100-200	1 vomited
	3	orally	300	Depression in 1
	4	orally	400-800	Vomiting in 2 (1 on 400 and 1 on 800, all recovered)

* The LD₅₀ was determined by graphic interpolation between the two values closest to 50% mortality.

2 Chronic toxicity Three rabbits were injected daily subcutaneously with 25 mgm/kgm and three with 50 mgm/kgm. AP 43 for 31 days. Three of the animals gained weight during the course of the experiment and the others lost slightly. There were no significant effects upon the blood picture, and urine examination was negative.

Histological examination was made on three animals of this group and no pathological change was seen in the hearts, kidneys, and livers.

A more extended chronic toxicity experiment was conducted on eight rats which were fed 25 mgm/kgm by stomach tube daily for seven weeks. None of the animals died and the average weight increased considerably. No effect upon the blood was noticed and urine examination was negative at the end of the experiment with the exception of a trace of albumin.

Two dogs weighing 4.5 kgm. each and one weighing 9.1 kgm. were injected twice daily subcutaneously with 100 mgm. of AP 43 for the period of three months. One of the small dogs occasionally had a short attack of convulsions after the injection. Otherwise, all dogs remained in good health and gained weight. Blood and urine examinations revealed normal findings. One of the animals was killed at the end of the experiment. There was no gross pathological change; a moderate degree of cloudy swelling was present in the liver and kidneys, fat stain of the liver was negative.

3. *Fate of AP 43.* Two dogs weighing approximately 6 kgm. each were injected subcutaneously with 150 mgm. AP 43. Urine was obtained by catheterization 3, 6, and 20 hours after administration and analyzed for AP 43 by the procedure described by Lehman and Aitken (2) for Demerol. The total excretion was 0.224 mgm. for one and 0.285 mgm. for the other dog; the last sample being practically completely devoid of the drug. This indicates that AP 43 does not appear in the urine to any significant extent.

A group of 12 rats was injected for three consecutive days with 0.75 cc./kgm. of carbon tetrachloride which produces a marked degree of liver damage. These rats showed no change in their sensitivity to AP 43 as the intraperitoneal convulsive and lethal dose was the same as in normal animals. Similarly, removal of both kidneys in rats did not influence the convulsive or lethal dose.

ANTISPASMODIC ACTION 1. *Effects on isolated smooth muscle.* The results obtained on isolated strips of rabbit intestine have been mentioned and are included in table 1. The action of AP 43 on spontaneous activity and upon acetylcholine and barium chloride-induced spasms is shown in figure 1.

Studies were also made on the isolated dog ureter, using essentially the same technique as given by Slaughter (3). Normal activity was sluggish with occasional contractions at one to three minute intervals. Addition of 1 cc. of 1/1000 Epinephrine to the 100 cc. bath increased spontaneous activity, with a marked increase in rate of contraction (fig. 2). Upon adding 10 mgm. AP 43, complete inhibition of activity resulted. After washing, spontaneous activity was once again restored. Similar results were obtained when 1 cc. of posterior pituitary extract (10 international units) was used to stimulate rhythmic contractions.

Excised strips of uterus from virgin rabbits were suspended in a 100 cc. oxygenated Ringer bath at 37.5°C. Introduction of 0.25 cc. of 1:2500 solution of posterior pituitary extract produced a temporary spasm. The addition of 10 mgm. AP 43 prior to pituitary extract reduced the spasm approximately one-half.

In other experiments, one horn of the guinea pig uterus was used. Addition of 2 micrograms of histamine phosphate to the bath resulted in a spasm lasting approximately 5 minutes. Introduction of 4 mgm. AP 43 at the peak of the contraction resulted in an immediate and complete relaxation. Doses of 1 mgm. AP 43 reduced the spasm by one-third. In comparison, 1 mgm. atropine reduced the spasm by 75% and 2 mgm. papaverine was equally effective.

2. *Action on the G.I. tract in vivo.* A. Stomach—Brucke, et al. (4, 5) reported

that the section of the vagi in the rabbit results in a spasm of the cardiac sphincter which could be relaxed by atropine, epinephrine, etc. These observations were confirmed in our experiments. Further work revealed that 0.5 to 1.5 mgm. of eserine injected intravenously would repeatedly produce a spasm and thus make possible antispasmodic studies on several drugs in one preparation. Rabbits were anesthetized with urethane. A tracheal cannula was inserted. A cannula was also put into the distal end of the esophagus and a second cannula placed in the stomach near the greater curvature. The cannula entering the esophagus was connected to a burette, which was in turn connected to a reservoir bottle, by means of which the burette could be filled to any desired level. Before each reading the burette was filled to the same point with saline from

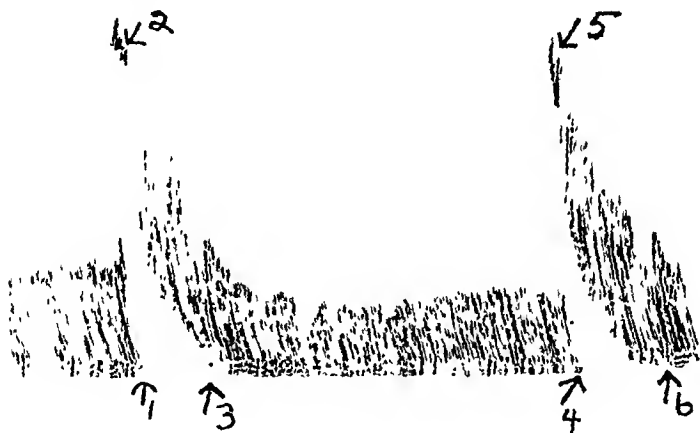


FIG. 1—A

the reservoir bottle, the bottle was clamped off, and the fluid from the burette allowed to run into the esophagus. The amount of fluid which ran out of the burette in a given time, either 0.5 or 1.0 minutes, determined the degree of spasm of the sphincter.

Following section of the vagi, which usually resulted in some spasm of the sphincter, an amount of eserine was injected intravenously to cause a complete spasm, the dose varying from 0.5 to 1.5 mgm. Then the antispasmodic was injected. The eserine dose was repeated after each dose of antispasmodic. Readings were taken approximately every three minutes. Since the duration of action of eserine itself is rather short, control experiments using eserine alone were carried out. It was necessary to increase the eserine dosage as the experiment progressed in order to obtain a full spasm. In three experiments 250 micrograms AP 43 was effective in relieving the eserine spasm. Atropine was effective in doses of 10 micrograms. 250 micrograms of Trasentin was

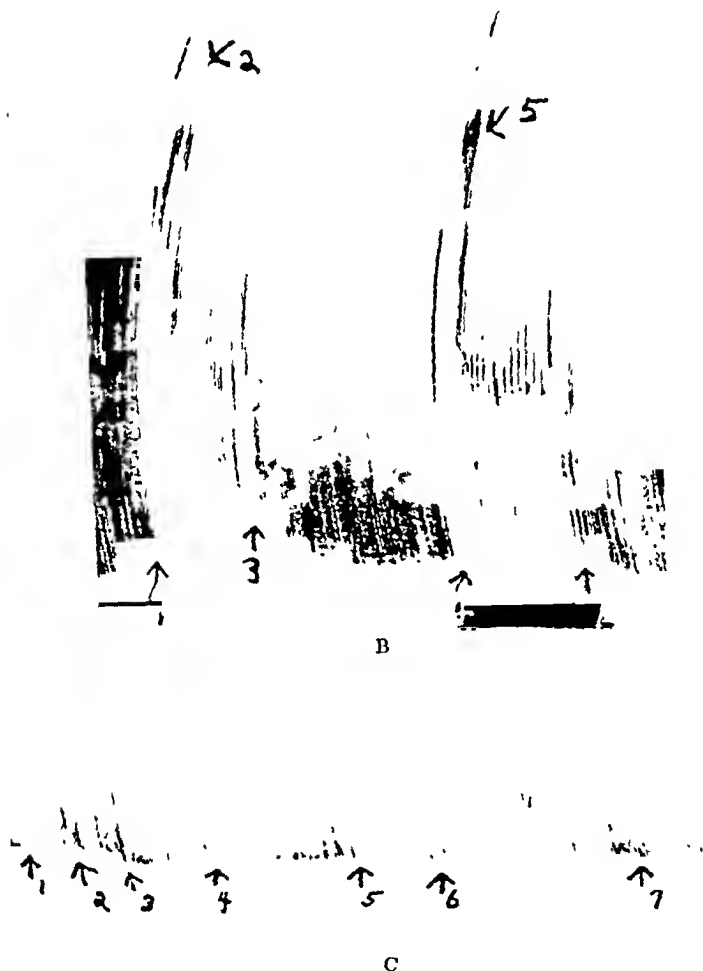


FIG. 1. ANTISPASMODIC ACTION ON ISOLATED RABBIT ILEUM
(Suspended in 100 cc. Ringer Bath)

- A (1) Acetylcholine, 20 gamma (2) AP 43, 10 gamma (3) Washed. (4) Acetylcholine, 20 gamma (5) Atropine, 1 gamma (6) Washed
 B (1) BaCl₂, 25 mg (2) Papaverine, 1 mg (3) Washed, fresh Ringer added (4) BaCl₂, 25 mg (5) AP 43, 0.5 mg (6) Washed
 C (1) AP 43, 1 mg (2) Washed (3) AP 43, 2 mg (4) Washed (5) AP 43, 5 mg. (6) Washed (7) AP 43, 3 mg

slightly less effective than AP 43, while papaverine in doses of 1 mgm. had no antispasmodic effect.

Observations of gastric motility were obtained on unanesthetized dogs having a total gastric pouch, in which the cardiac end of the stomach had been resected and closed and the pyloric opening brought to the outside. Continuity of the intestinal tract was restored by anastomosis of the cardiac end of the esophagus to the side of an upper jejunal loop. In two such dogs, a balloon was inserted and stomach activity recorded with a water manometer. Injection of 2.5 mgm. AP 43 reduced normal tone slightly, and 12.5 mgm. AP 43 greatly reduced peristalsis and stomach tone. As shown in figure 3, the intravenous injection of 0.75 mgm. arecoline hydrobromide produced an intense spasm lasting several minutes. Ten minutes later, an injection of 12.5 mgm. AP 43 followed in five minutes by 0.75 mgm. arecoline resulted in only a very slight spasm. Twenty

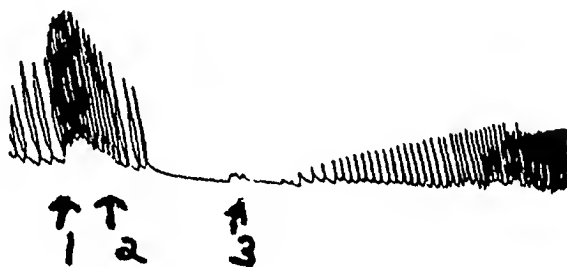


FIG. 2. THE EFFECT OF AP 43 ON URETER

Excised dog ureter in 100 cc. Ringer bath. (1) Addition of 1 cc. of 1:1000 Epinephrine. (2) Addition of 10 mg. AP 43. (3) Washed.

minutes after the AP 43, injection of 0.75 mgm. arecoline once again produced a marked spasm.

B. Small Intestine—A comparative study of the antispasmodic drugs was made in five dogs having Thiry Vella fistula loops. A total of 20 experiments were made. Tracings of normal activity showed continuous marked peristalsis. Injection of 2.5 to 10 mgm. of AP 43 reduced or completely abolished activity. Standardization tests revealed that arecoline was the most useful spasmogenic agent, since the spasm produced occurred promptly and was highly reproducible. Dosage was 0.5 to 1.5 mgm. given intravenously, depending on the weight of the animal. These dogs ranged from 10 to 25 kgm. After injection the dogs showed signs of restlessness, salivation and retching. On two occasions a short convulsive seizure was observed. A typical tracing is shown in figure 4. Within 10 seconds of the injection, a pronounced spasm of the intestine resulted, which persisted for two to three minutes with a gradual return of normal activity.

Injection of 10 to 30 mgm. AP 43 at the peak of the spasm caused an immediate drop in tone to normal levels. When AP 43 was injected before arecoline, no or only a slight spasm was observed. Excitement and retching were also abolished. With this type of experiment, one may estimate the duration of action of AP 43 by repeating injections of arecoline at intervals after a single dose of the antispasmodic and determining the time at which a full arecoline spasm can again be obtained. It was found that a dose of 10 mgm. AP 43 maintained

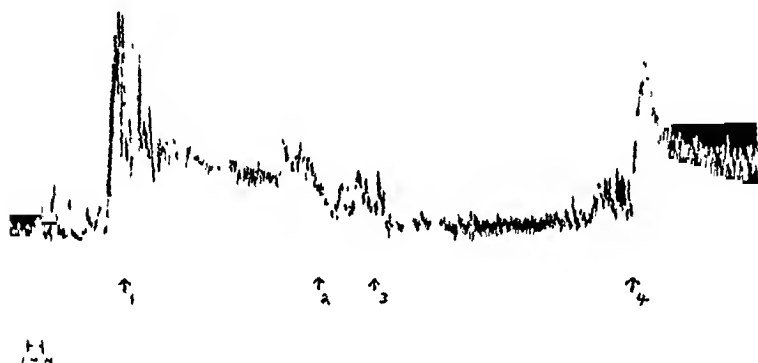


FIG 3 UNANESTHETIZED DOG TOTAL STOMACH POUCH BALLOON RECORDING
All injections intravenously. (1) 0.75 mg arecoline. (2) 20 mg AP 43. (3) 0.75 mg. arecoline (4) 0.75 mg arecoline.

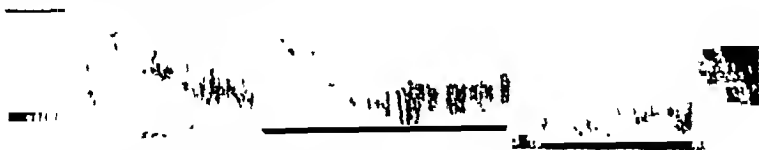


FIG 4 THIRY VELLA FISTULA DOG BALLOON RECORDING
All injections intravenously (1) 1 mg arecoline (2) 1 mg. arecoline. (3) 15 mg. AP 43 (4) 1 mg arecoline (5) 1 mg arecoline.

its antispasmodic action for approximately 30 minutes. Similar spasmolytic activity was observed with AP 73 and atropine. However, atropine is more powerful; the effective dosage was 0.5 to 1 mgm. Comparative evaluation of these three drugs shows that atropine has 10 to 20 times the spasmolytic activity of AP 43 and AP 73. With appropriate dosage, however, they were all highly effective in antagonizing arecoline spasms.

Studies were also made on three decerebrate cats using the Babkin method (6) for recording intestinal activity. The animals were immersed in a constant temperature saline bath and segments of the intestine in vivo were cannulated and filled with sesame oil. Activity was recorded by means of a water manom-

eter. In these studies it was found that 1.5 mgm. AP 43 would reduce or prevent the spasm induced by 0.1 mgm. arecoline in much the same way as observed in the Thiry Vella dogs.

C. Colon—In experiments on three double fistula dogs (both Thiry Vella and colon fistulas) simultaneous records were obtained of the small intestine and colon activity. The injection of 1 mgm. arecoline causes a marked spasm in both small intestine and colon lasting approximately five minutes, as shown in figure 5. The injection of 10 mgm. AP 43 followed in one minute by 1 mgm. arecoline reduced the spasm to approximately one-half in the small intestine and to one-fourth or less in the colon. Some spasmolytic action is still present in the colon response to arecoline twenty minutes later. A full spasm in both



FIG. 5 UNANESTHETIZED DOG, SIMULTANEOUS BALLOON RECORDING FROM COLON AND THIRY VELLA FISTULAE. INTRAVENOUS INJECTIONS
(1) 1 mg. arecoline. (2) 10 mg. AP 43 (3) 1 mg. arecoline. (4) 1 mg. arecoline. (5) 1 mg. arecoline.

small intestine and colon was again obtained 35 minutes after AP 43. In other experiments, four dogs with cecostomies were used. Spasm was induced by means of 0.75 to 1.5 mgm./kgm. morphine injected subcutaneously. This resulted in a marked sustained tone which was partially reduced by 15 mgm. of AP 43 (see fig. 6). AP 73 in an equal dose produced similar effects. Atropine was also only temporarily effective in reducing the spasm. The first injection of any of these antispasmodics after establishment of the morphine spasm was usually effective. However, after the spasm had recurred, further administration of any of these drugs was much less effective. Quantitative evaluation of antispasmodic activity in such experiments is thus difficult. Atropine, however, was usually the most reliable in temporarily relaxing the spasm. The difficulty in obtaining an adequate antispasmodic action against morphine spasm of the dog colon has also been observed and emphasized by Atkinson et al. (7).

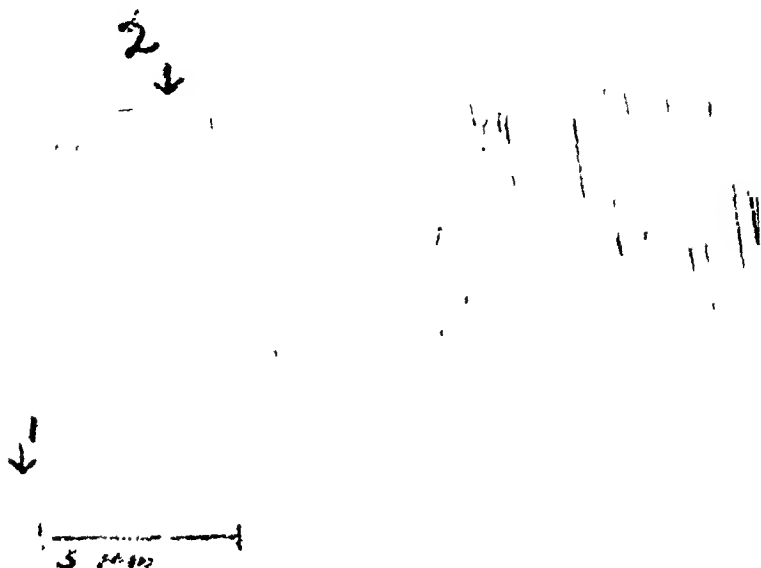


FIG. 6. COLON FISTULA DOG

(1) 0.75 mg./kg. Morphine subcutaneously. (Drum stopped for ten minutes until spasm established. (2) 15 mg. AP 43 intravenously.

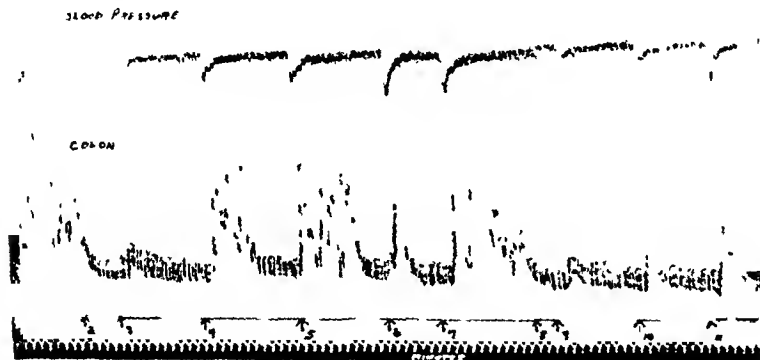


FIG 7 DOG, NEMBUTAL ANESTHESIA, BLOOD PRESSURE FROM CAROTID ARTERY BALLOON RECORDING OF DESCENDING COLON ALL INJECTIONS INTRAVENOUSLY

(1) 0.5 mg arecoline (2) 10 mg AP 43 (3-7) 0.5 mg arecoline at each injection. (8) 10 mg AP 43 (9-11) 0.5 mg arecoline at each injection

In two experiments, dogs under Nembutal anesthesia were prepared for blood pressure recording from the carotid artery, and a balloon was inserted into the lower colon via the rectum. As shown in figure 7, the injection of 0.25

mgm./kgm. arecoline intravenously caused a marked colonic spasm and a fall in blood pressure of 50 mm. Ten minutes later, 10 mgm. of AP 43 were injected slowly. No change in the tone of the colon or blood pressure occurred. Then 0.25 mgm./kgm. arecoline was injected. No spasm appeared in the colon, but blood pressure fell 40 mm. Twenty minutes later, another injection of 0.25 mgm. arecoline was given, which resulted in a colon spasm and fall of 50 mm. in blood pressure. A second injection of 10 mgm. AP 43 resulted in a pronounced antagonism of arecoline spasm for 30 minutes.

Three dogs were starved for 24 hours and given an enema to eliminate fecal matter. Then, under Nembutal anesthesia, they were prepared for study of gaseous pressure changes in the colon.³ This was done by applying a rubber



FIG. 8. RELAXING ACTION OF AP 43 ON AIR INFLATED COLON
Dog, light nembutal anesthesia (see text for method). At 1, 20 mg. AP 43 injected.

pad to the anus with rubber cement, so that no gas could escape. The colon was then distended with 200 cc. of air by a rubber tube fixed in the center of the glued pad. Pressure changes were recorded by means of a water manometer. Figure 8 is typical of the activity in the colon after the injection of air. Intravenous injection of 20 mgm. AP 43 caused a marked decrease in activity and pressure, which is indicative of relaxation of the colonic musculature. Twenty minutes after injection of the antispasmodic, normal activity returned abruptly.

ACTION ON UTERUS IN SITU. The effect of 100 mgm. AP 43 given intravenously on the uterine contractions in a severe case of dysmenorrhea is shown in figure 9. The method used has been described by Bickers (8). Similar records were obtained with intramuscular injection, but the onset of antispasmodic

³ This method was developed by Dr. F. R. Steggerda, Dept. of Physiology, University of Illinois, Urbana, Illinois.

effect was slower. With the cessation of rhythmic contraction the patient was completely relieved of painful symptoms.⁴

LOCAL ANESTHETIC AND IRRITATION TESTS. Topical local anesthetic action was determined by instilling 1 cc. of a 1% solution into the rabbit's eye and at intervals of 1 to 5 minutes testing for the wink reflex by touching the cornea with a probe. Duration of anesthesia and degree of irritation of the benzofuranones are recorded in table 1. AP 43 showed prompt marked local anesthetic action. In 10 tests the reflex was abolished for 30 to 80 minutes, compared with 30 to 40 minutes for Butyn sulfate. In equal concentration the latter was somewhat less irritating. Two tests in human subjects revealed that instillation into the eye of a 1 or 2 per cent solution of AP 43 (buffered to pH 7) caused transitory pain, until anesthesia set in.

Further studies for local anesthetic action were made using a modification of the guinea pig wheal test described by Rose (9), in which the animals are given 20 mgm./kgm. nembutal i.p. as a light hypnotic. This prevents the development of a conditioned reflex to the buzz of the inductorium but does not interfere with the muscle reflex or vocal response to effective stimuli. The action of

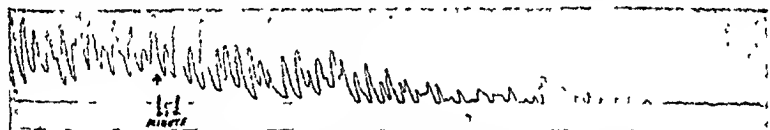


FIG 9 SPASMOLYTIC EFFECT OF AP 43 ON HUMAN UTERUS

Uterine contractions of a woman with severe dysmenorrhea. Balloon recording. At arrow, 100 mg AP 43 given intravenously

local anesthetics is somewhat prolonged with this method but by using procaine as a standard in each animal, comparative values for the drugs tested can be determined. 0.5 cc. of a 1% solution of AP 43 was injected intradermally and compared with equal concentrations of procaine HCl. AP 43 was found to have a prompt local anesthetic action lasting 60 to 80 minutes, as compared with 40 to 55 minutes for procaine. The site of injection of AP 43 showed moderate tissue irritation. Intradermal tests made in humans revealed that the injection of 0.5% of AP 43 resulted in a brief disagreeable initial stinging sensation before local anesthetic action became manifest. In two human subjects the duration of anesthesia at the site of injection of 0.07 cc. of 0.5% solution was 41 and 42 minutes for AP 43 and 18 and 22 minutes for procaine HCl. AP 43 was slightly more irritating, but no tissue damage resulted.

Further studies using the sciatic nerve block method of Shakell (10) in 6 guinea pigs showed that 0.25 cc. of a 1% solution of AP 43 induced anesthesia lasting 59 to 100 minutes as compared with 23 to 24 minutes for two procaine controls. There was no evidence of damage to the nerve, since the transitory motor paralysis disappeared together with the anesthesia. Autopsy of rabbits

⁴ The authors are indebted to Dr Wm Bickers for this observation and the permission to use figure 5

24 hours after an intramuscular injection of 1 cc. of a 5% solution showed complete absorption with no significant tissue damage apparent. Subcutaneous injection of this solution resulted in transitory local edema.

STUDY OF SIDE REACTIONS. *Salivary secretion.* One of the disagreeable side effects resulting from therapeutic doses of atropine is a marked drying of the mouth which results from an inhibition of salivary secretion. Studies were undertaken to see how AP 43 and AP 73 compared with atropine in regard to this effect. These experiments were made on rabbits given 1 gram/kgm. of urethane by mouth as a sedative. Salivary secretion was stimulated by the intramuscular injection of 5 mgm./kgm. pilocarpine HCl, and the saliva volume was measured at half-hour intervals for two hours. A few days later,

TABLE 3
Antagonism of salivary secretion by AP 43, AP 73 and atropine

NO. OF RABBITS	ANTISPASMODIC	DOSE	PILOCARPINE	AVERAGE SALIVARY VOLUME PER ANIMAL/ HOUR
		<i>mgm./kgm. i. m.</i>	<i>mgm./kgm.</i>	
5	Control		5	20.0
8	Control		5	30.0
8	Control		5	28.0
5	Atropine	0.3	5	0.9
6	Atropine	0.5	5	1.2
8	Atropine	1.0	5	3.0
6	Atropine	3.0	5	6.5
11	AP 43	3.0	5	28.1
4	AP 43	3.0	5	26.0
11	AP 73	3.0	5	26.0

the same rabbits were again given the previous dosage of urethane and pilocarpine plus a simultaneous intramuscular injection of 3 mgm./kgm. of the antispasmodic. The results are summarized in table 3.

From these data it is evident that AP 43 and AP 73 in a dose of 3 mgm./kgm. have no inhibitory action on the rate of salivary flow induced by pilocarpine. In contrast, atropine exerted a profound inhibitory effect, reducing the salivary flow by 90%, even with doses 10 times less than AP 43 or AP 73.

Mydriatic action. Pupillary dilatation is another side effect of atropine which limits its usefulness as an antispasmodic. Studies were undertaken to compare this series of antispasmodics with atropine. In cats under light Nembutal anesthesia, gradually increased doses of these drugs and atropine were injected intravenously until a measurable pupillary dilatation was observed. In all instances the pupils were exposed to a constant light source at a distance of 12 inches from the eyes. A study of table 4 shows that AP 43 is approximately 100 times less active as a mydriatic agent than atropine. Topical myd-

riatic effect obtained by instillation was also determined in cats. Several instillations of a solution of 1 part in 100,000 of atropine resulted in a dilation from a normal of 1 mm. to a horizontal diameter of 3 mm. A 1:10,000 solution caused dilation from a normal of 2 mm. to 8 mm. in one hour. A 1:100 solution of AP 43 caused dilation from 1 mm. to 5 mm. diameter, while a 1:20 solution of AP 43 caused no greater effect. A 1:50 solution of AP 73 also caused a discernible dilation.

Cardiovascular effects. AP 43 and AP 73 show a brief vasodepressor action. In doses of 1 or 2 mgm./kgm., a transitory fall in blood pressure of 40 to 60 mm. occurred in 3 cats and 5 dogs under Nembutal anesthesia. The magnitude of the blood pressure drop is largely dependent on the rate of injection. These falls in blood pressure occur in atropinized or vagotomized animals, therefore, the effect is not mediated through the parasympathetic nervous system. Two

TABLE 4
Mydriatic activity of AP 43 and atropine

NO CATS	DRUG	MG/M/KGM (INTRAVENOUSLY)	AVERAGE CHANGE IN PUPIL SIZE IN MM
1	AP 43	1 0	0
2	AP 43	2 0	3
2	AP 43	2 5	2.0
1	AP 43	3 0	3.5
1	Atropine	0 005	0 5
3	Atropine	0 01	0.5
4	Atropine	0 02	1.5
1	Atropine	0 03	3 0
1	Atropine	0 05	2 0
1	Atropine	1 0	3 0

experiments on the perfused rabbit's ear clearly showed the peripheral vasodilator action of AP 43. The rate of flow increased 40% with injection of 5 mgm. AP 43. Injection of 1 microgram epinephrine resulted in a vasoconstriction and reduced flow approximately 35 to 50% below normal. Simultaneous injection of 1 microgram epinephrine and 5 mgm. AP 43 resulted in no change from normal indicating a mutual antagonism between the two drugs on the peripheral vessels.

It is well known that atropine paralyzes the parasympathetic nerve endings so that stimulation of vagi or injection of acetylcholine no longer produce a vasodepressor response. This action manifests itself clinically as an undesirable tachycardia. Three dogs under Nembutal were prepared for carotid blood pressure recording. The vagi were severed and the distal ends stimulated electrically. Voltage was varied by means of a Harvard inductorium. After establishing the voltage necessary to produce a 30 to 50 mm. fall in pressure, the drugs were injected intravenously and then vagal stimulation was repeated. With doses of 200 micrograms/kgm. AP 43, no inhibition of vagal action was

noted. However, 10 micrograms/kgm. atropine caused a marked decrease in vagal effect even with the voltage increased to double the original value. Because of the difficulties in getting constant responses to stimuli of the same intensity, it was not possible to obtain comparative data in every test. However, atropine in all instances was definitely more inhibitory than AP 43. Experimental studies with the method of Fromherz (11) using injections of acetylcholine gave highly consistent results and made more accurate comparisons possible. A typical tracing is shown in figure 10. It is seen that 200 micrograms/kgm. AP 43 has a short inhibiting action on acetylcholine response, equivalent to the action of 2 micrograms atropine. Thus, atropine is approximately 100 times more inhibitory than AP 43. AP 73 was found to be similar to AP 43. Studies of three Straub frog heart preparations revealed that 1:1000 of AP 43 caused a gradual slowing of the heart with final cessation of ventricular beats with a tendency to systolic contracture. The auricles continued to beat at a much reduced rate for a while. At this stage reversibility was only partial with some return of auricular and ventricular activity, but still at a rate far less than normal.

Electrocardiographic studies were made in two dogs. After obtaining a normal tracing, under 25 mgm./kgm. Nembutal, 10 mgm./kgm. AP 43 were injected intravenously. The heart rate increased from 126 to 144 per minute. The only observable change in the record was a slightly more positive T wave in lead II. Studies were also made with the Hamilton manometer. Injection of 2 mgm./kgm. AP 43 intravenously in an unanesthetized dog caused a transitory fall in blood pressure of 20 mm. No significant change in heart rate was noted.

ANTI-HISTAMINE ACTION. Since AP 43 was effective against barium chloride-induced spasm and antagonized the histamine contraction on the isolated guinea pig uterus, its possible usefulness as a bronchio-dilator was studied. In these experiments, Tainter's (12) modification of the method given by Sollmann and von Oettingen was used. The rate of flow of the perfusion fluid was used as an index of bronchio-constriction or dilation. Spasms of the bronchi were induced by injecting 50 to 500 micrograms histamine phosphate into the perfusion cannula. The resulting spasm was maintained for periods of 20 to 30 minutes. However, the injection of 1 to 5 mgm. of AP 43 as soon as the histamine effect was maximal resulted in a marked sustained bronchio-dilator action. In comparative experiments, AP 43 was more effective than an equal amount of theophylline. Epinephrine was an effective bronchio-dilator with doses of 50 to 100 micrograms.

The antagonism of anaphylactic shock was studied in 12 guinea pigs, which were sensitized by the intraperitoneal injection of .1 cc. of human serum. Twenty-one days later, 6 of the pigs were given .2 cc. of human serum intracardially. Five of the 6 showed marked anaphylactic symptoms, and 3 of the group died. In the other 6 pigs, 20 to 30 mgm. of AP 43 was injected slowly intracardially, followed in 5 minutes by the injection of .2 cc. of human blood serum. Marked symptoms were seen in only one animal, which died. The other 5 showed but

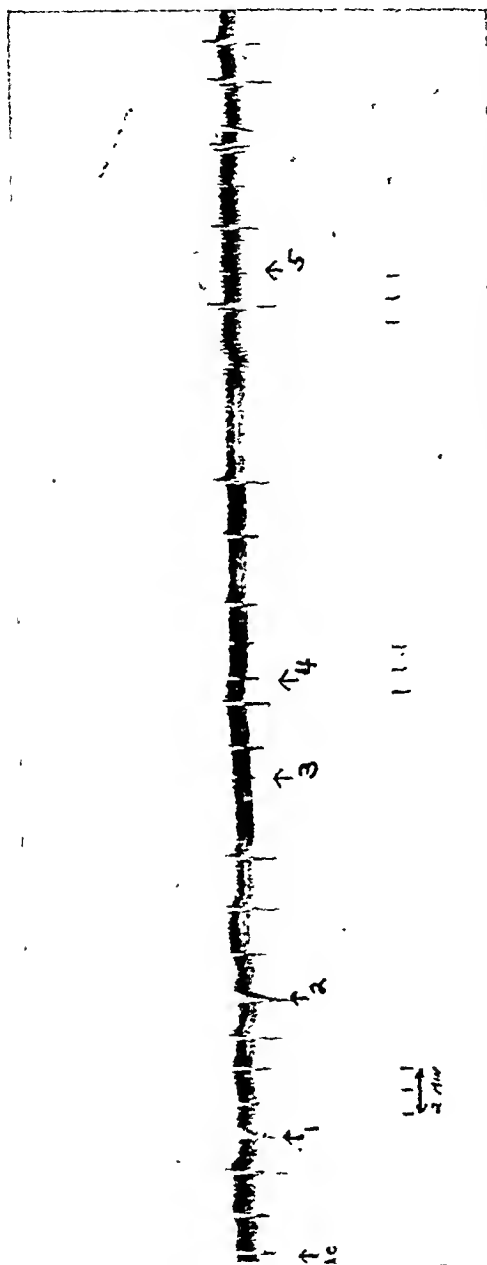


FIG. 10. COMPARISON OF THE INHIBITORY ACTION OF ATROPINE AND AP 43 ON THE BLOOD PRESSURE RESPONSE TO ACETYLCHOLINE. CAT, NEMBUTAL ANESTHESIA. ALL INJECTIONS MADE INTRAVENOUSLY

(A C.) 1 microgram acetylcholine injected every 2 to 5 minutes throughout experiment. (1) 0.1 mg./kg. AP 43. (2) 0.2 mg./kg. AP 43. (3) .002 mg./kg. Atropine. (4) .004 mg./kg. Atropine. (5) .004 mg./kg. Atropine.

slight symptoms and recovered. The intracardial injection of 7 mgm./kgm. AP 43 prior to an established fatal dose of histamine phosphate (0.4 mgm./kgm.) prevented histamine shock in 2 guinea pigs. Symptoms of bronchial spasm were not observed. Blood pressure studies in three cats under Nembutal revealed a transitory antagonistic action of AP 43 against the vasodepressor effect of histamine. Injection of 1 microgram of histamine caused a fall in blood pressure of 50 mm. while one minute after 2 mgm./kgm. AP 43, 1 microgram of histamine had no effect. One mgm./kgm. AP 43 reduced the histamine action by one-half. This action is short-lived, since full histamine depressor action is present again within 5 to 10 minutes after AP 43. From these experiments, it appears that AP 43 has a general antagonistic action against histamine effects. However, it is difficult from the data at hand to evaluate this action in comparison with other drugs.

DISCUSSION AND SUMMARY. A variety of methods have been used in this study with the idea of characterizing the pharmacological action of these benzofuranone derivatives and to make comparisons with such well-known drugs as atropine and papaverine.

The preliminary tests using isolated segments of rabbit intestine have been found useful in determining antispasmodic activity. Although the use of these *in vitro* tests have limitations due to the artificial conditions imposed on the muscle, such experiments have in this series given a reliable index of antispasmodic activity. This is clearly indicated by the close correlation of the relative effectiveness of the drugs against acetylcholine in these tests, with the values obtained in *in vivo* experiments using arecoline.

Among the most reliable tests for antispasmodic activity are those made on fistula dogs. Since anesthesia is not necessary, the possible synergistic action of hypnotics with antispasmodic drugs is avoided. Further, the other symptoms produced by arecoline and the antagonistic action of the antispasmodic drugs on these can also be observed.

The necessity of inducing a spasm which may be repeated, has lead to the use of several drugs for this purpose. Mecholyl has been used by Clark et al. (13) in Thiry Vella fistula dogs. In the present report, arecoline has proven highly satisfactory as a spasmogenic agent in studies of the stomach, intestine and colon. This drug, like pilocarpine, is believed to act on those smooth muscles which are innervated by cholinergic fibers (see Dixon (14)). Its advantages are the constancy of the spasm produced and its short duration (approximately five minutes). In spasmogenic doses it causes some signs of central stimulation such as excitement, but these reactions are seldom so severe as to interfere with the experiment. By giving both the arecoline and the antispasmodics intravenously, variables of absorption and destruction by tissues at the site of injection are eliminated.

In studies on colon fistula dogs, morphine was used to induce a spastic colon. This method is generally unsuitable for accurate appraisal of antispasmodics for several reasons. First, the spasm induced by morphine is prone to wide and variable fluctuations of tone. Second, the spasm is very difficult to break

by antispasmodic drugs in doses which do not cause excessive side reactions, i.e., excitement, convulsions, etc. Third, the response to the same drugs may vary during the course of the experiment, usually being less effective on succeeding injections.

Throughout these studies on the gastro-intestinal tract there was a generally consistent ratio of activity, the effective dose of AP 43 being approximately 10 to 15 times that of atropine.

The powerful local anesthetic action of some of these benzofuranones is a property shared by a variety of antispasmodic drugs, Crohn, et al. (1); Lehmann and Knoefel (15, 16). The possible importance of the topical anesthetic action of antispasmodics when administered orally is particularly emphasized by the work of Crohn, et al. (1). However, in the present study it may be noted from table 1 that there is no evidence of a strict correlation between local anesthetic and antispasmodic activity.

The four most distressing side reactions of atropine in therapy are central excitement, mydriasis, tachycardia (due primarily to a loss of vagal effects), and xerostomia resulting from the inhibition of salivary secretion.

In the mice the symptomatology and MLD_{50} of atropine and AP 43 are similar. They both act on the central nervous system to produce excitement and convulsions. In dogs, the CNS effects of AP 43 are alleviated by non-sedative doses of Nembutal. Concerning the other side reactions, the experiments on mydriatic activity indicate AP 43 is approximately 100 times less active than atropine. The same ratio was found for the inhibition of acetylcholine and vagal effects on blood pressure in cats and dogs. If one compares the antispasmodic activity of AP 43 and atropine to the side reaction activity of these drugs, they are in a ratio of ten to one in favor of AP 43 not producing side reactions in therapeutic doses.

The studies on isolated guinea pig lungs showed that AP 43 is effective against histamine spasm. This is supported by *in vivo* experiments in which AP 43 protected the animals against histamine and anaphylactic shock.

Preliminary clinical trials indicate that AP 43 possesses antispasmodic activity in humans. Prince and Richardson (17) have recently demonstrated its efficacy in the treatment of ureteral and bladder spasms. Side effects have been chiefly referable to central stimulation.

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Addenda: "Amethone" has been tentatively approved as a trade name for AP 42.

CONCLUSIONS

1. A new chemical series, the benzofuranones, have been investigated and shown to have antispasmodic action. Among the best of the compounds, 3-

(beta-diethylaminoethyl)-3-phenyl-2-benzofuranone HCl (AP 43) shows approximately $\frac{1}{10}$ to $\frac{1}{15}$ the activity of atropine against acetylcholine and twice the activity of papaverine against BaCl₂ spasm of isolated rabbit ileum.

2. The duration of local anesthetic action of AP 43 is equal or superior to Butyn and Procaine.

3. Studies in dogs with stomach, Thiry-Vella and colon fistulae using arecoline as a spasmogenic agent showed AP 43 to have approximately $\frac{1}{10}$ the antispasmodic activity of atropine. AP 43 also temporarily reduced morphine spasm of the colon in dogs.

4. AP 43 was effective against histamine spasm of perfused guinea pig lung preparations and also reduced or prevented symptoms of anaphylactic shock when given prior to the shocking serum.

5. In comparative studies of side reactions, AP 43 shows only a fraction of the activity of atropine as a mydriatic on the pupil, on the inhibition of salivation, and the inhibition of vagal and acetylcholine action on blood pressure.

6. The methods and their usefulness are evaluated.

7. Clinical trials have confirmed the antispasmodic activity of AP 43 in humans.

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